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Attempted Long-Term Culture of Human Bronchial Mucosa and Bronchial Neoplasms[1, 2, 3]

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Summary

Fragments of adult human bronchial tissue have been maintained in tissue culture for long periods. The epithelial mucosa may retain its ciliary activity for as long as 135 days when grown on perforated cellophane, and not submerged, in a medium composed of 20 percent human placental serum, 40 percent medium 199, and 40 percent Tyrode's solution containing added antibiotics. Some mucus material is retained in the mucous glands for as long as 3 weeks; however, evidence is not available as to whether secretory activity persists. In contrast to the behavior of normal ciliated epithelium, bronchogenic (chiefly squamous-cell) carcinomas have resisted not only propagation but also attempts at long-term survival, by tissue-culture techniques. Histological sections of tissue from bronchogenic carcinoma show entrapped areas of normal ciliated epithelium. Survival and outgrowths of cells from this normal tissue have been noted frequently during unsuccessful attempts to culture neoplastic cells.—**J. Nat. Cancer Inst. 22: 1027–1037, 1959.**

Explants from human adult lung tissue and bronchogenic carcinoma, grown in tissue culture, were utilized as part of a long-range program designed to study various physical and chemical influences which might be important in the pathogenesis of bronchogenic carcinoma. Therefore the investigation had a dual objective: 1) to obtain epithelial cell lines from outgrowth of normal and neoplastic bronchial tissue, and 2) to study the degree to which the explant remained viable, functional, and histologically identical with similar tissue *in vivo*.

Materials and Methods

Samples of bronchus, bronchiole, and tumor were obtained from lung tissue, under as aseptic conditions as possible, within a few minutes after their surgical removal. Pieces for culture were washed in Tyrode's

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[3] Part of this work was presented in preliminary form at the meeting of the Tissue Culture Association in Philadelphia, Penna., April 10, 1958.

solution containing penicillin and streptomycin, and then rinsed several times (also in Tyrode's solution) to remove clotted blood. Small fragments (about 1 mm. thick and 2 to 4 mm. square) were cut from the tumor tissue with a fine, sharp scissors. The larger pieces of bronchus were pinned to a large rubber stopper (#8–9) and thin strips of mucosa were dissected away with a sharp scalpel or cataract knife. Smaller bronchi were trimmed free of lung tissue, and segments were used as full-thickness explants.

Samples of fresh tissue were fixed in Bouin's fluid, and sections were stained with hematoxylin-eosin and periodic acid-Schiff reaction. At intervals of 3 to 7 days, throughout the experiments, random cultures were fixed for histological study of the explant as well as for cytological observations on the outgrowth.

Over a 2-year period, many variations in media and technique were tried. Preliminary experiments utilized the classic technique of clot cultures in Carrel flasks, which was soon abandoned because of the rapid lysis of the clot produced by most of the explants. The use of coverslips, with or without the addition of a small chick-plasma or an agar clot as substrate for the explant, gave slightly improved cultural conditions.

Of all the combinations used, best results were obtained with explants placed on perforated cellophane in Carrel flasks or Earle's T-30 or T-60 flasks. Small volumes of media were added so that the tissue was bathed and moistened by the liquid, but not submerged. Medium volumes were 0.5 to 0.6 ml. for a D-3.5 Carrel flask and 3 to 3.5 ml. for a T-30 flask.

Media were composed basically of 20 percent serum, 40 percent medium 199, and 40 percent Tyrode's solution, with 50 units of penicillin and 50 μg. of streptomycin per ml. Various serums were used: human adult, human placental, horse, lamb, calf, and chicken. In certain media, human ascitic or pleural fluid at a concentration of 40 percent was used in place of serum, with a proportional reduction in the Tyrode fraction of the medium. A number of additives were also tried: 50 percent chick-embryo extract (EE50), yeast extract, peptone, human albumin and globulin, and Simm's Z-16 salt solution (*1*).

Exploratory trials were made of several, quite different, culture methods. Tumor cells dispersed with trypsin were planted in gradient culture bottles—this was similar to the method described by Osgood and Krippaehne (*2*). Tissue pieces were placed on the surface of clotted media, with and without support of rayon or lens paper, in an approach to the method described by Fell (*3*).

Since neither of the latter 2 methods was used extensively, final conclusions were not reached as to their ultimate value in studies of this type.

Results

Most satisfactory results, both from a visual and histological view, were obtained with perforated cellophane. Outgrowth, when present, could easily be seen, and the ciliary activity or mucus secretion of the

bronchial mucosa could be observed over relatively long periods (2 weeks to several months).

Media containing human placental serum, or certain lots of ascitic fluid, gave the best results. The use of Simm's Z-16 solution at a concentration of 20 percent in the medium seemed beneficial to epithelial outgrowth. Chick-embryo extract, whether freshly prepared or stored at 4° C., had no apparent effect on the cultures.

The results obtained with bronchial tissue are presented in table 1.

Bronchial Mucosa

During the first few days of culture there was a general sloughing of cells, often providing a rotating globe of hundreds of epithelial cells, as illustrated by Corssen and Allen (*4*), which continued to spin around in the medium for several days. Mucus was secreted into the medium at this time. The intact epithelium, when showing ciliary beating, retained this function for long periods, often 60 days or more, and, in one extreme instance, ciliary action was observed for as long as 135 days. Sections of these explants showed that the ciliated cells were often present in only a single layer over the explant which otherwise was fibrous and nearly acellular. Prolonged retention of ciliary differentiation was noted only in cells that were in contact with the connective tissue underlying the original epithelium. Cells that had migrated over adjacent tissues such as cartilage did not possess cilia. Mitosis was observed in the epithelial cell layer in tissue that had been cultured 10 to 20 days. At this time the layer was usually 2 to 3 cells thick, and the mitoses were noted in cells nearest the basement membrane.

The pseudostratified ciliated columnar epithelium (5 to 6 cell layers in thickness, fig. 1) became thinner; by the end of 7 to 10 days, the mucous membrane was frequently only 2 to 3 cells thick, except in certain invaginations and/or glandular ducts not exposed to the surface, which occasionally showed nearly normal columnar appearance and cilia after 4 or more weeks of culture (fig. 2). By the end of 2 to 4 weeks the epithelial layer was usually only 1 to 3 cells thick (fig. 3) and in many explants abundant epithelial outgrowth, frequently accompanied by fibroblast outgrowth, was observed. Characteristically, the outgrowths of epithelial cells never penetrated into or under the perforations of the cellophane, but always edged around them. When an explant straddled a perforation, no outgrowth occurred at the epithelial edge. Conversely, fibroblastic outgrowth, when it occurred, spread through the flask, above and below the cellophane and on the glass.

The mucous glands showed rather rapid degenerative changes from the fresh state (fig. 4). Flattening of the cells occurred generally within 2 to 3 weeks (fig. 5).

Tumor Tissue

Five squamous-cell carcinomas, 2 undifferentiated carcinomas, and 1 metastatic sarcoma (table 2) were studied extensively, and over 100

TABLE 1.—*Reaction and outgrowth of bronchial tissue in various media*

Tissue No.	Substrate* and media†	Number of cultures	Types of cells‡ and outgrowth observed			Comments
			1st week	2 to 4 weeks	1 to 3 months	
Bronchial 1	I As Co Ia CoEE50 HuEE50	20	3B, 2E, 3F	1B, 1E	— lysed	Explants lysed off clots and rounded up. Older cultures showed mostly fibroblasts.
2		12	1E, 2F, 3M	2F	— lysed	
3		8	1B, 1F, 3M	3E, 1FE	4F, 1M	
4		15	4B, 2E, 3F	1B, 4E, 2F, 2M	3E, 4F	
5		11	6B, 1E, 1F	Poor growth—lysis in all		
Bronchial 6	I, II CoEE50 II HuEE50	13	6B, 3E, 1F	2B, 5E	1B, 2E	One epithelial sheet maintained 6 weeks. Mitosis seen in outgrowth; beating cilia observed 135 days.
7		13	6B, 2E	3B, 4E, 1M	1B, 2EF, 1F	
Bronchial 10	As II and Co	20	3B	6E, 1F	2EF, rest fixed and stained	Abundant E cells during 1st month, then overgrown by F. No noticeable differences in As and Co media.
11		8	4B	4E, 1F	fixed and stained	
13		12	7F	1EF, 7M	4F, 1EF, 1M	Many giant multinucleate cells in mixed cultures with F.
Bronchial 14	II CoEE50 III CHEE50	4	4B, 2E	4B	1B	Ciliary beating maintained 100 days. Mitosis seen in basal layer 10 to 15 days.
		13	No good visual observations—fixed and stained			
Bronchial 15	III CoEE50 CHEE50	9	No visual observations—fixed and stained			Ciliated E in 5-day fixed cultures; rapid necrosis by 9th day.

*Substrate: I = plasma clot; Ia = agar clot; II = perforated cellophane; III = organ-type culture (after Fell).

†Media: As = 40% ascitic fluid, 40% 199, 20% Simm's Z-16; CHEE50 = ¾ chick plasma, ¼ EE50; Co = 20% cord serum, 40% 199, 20% Simm's Z-16, 20% Tyrode's; CoEE50=40% cord serum, 40% 199, 20% EE50; HuEE50 = 20% human serum, 40% 199, 20% EE50, 20% Tyrode's.

‡Cells: B = ciliary beating; E = epithelial cells; F = fibroblast cells; M = mixed outgrowth of E or F and some large granular, multinucleate cells.

cultures and subcultures were made. Histological sections from contiguous portions of the fresh material used in the cultures demonstrated that the cellular, healthy margin of the tumor had been explanted. Although inflammatory and/or necrotic cells in considerable numbers were frequently found, abundant tumor cells were always present in the fresh material. Tumor explants usually showed rapid degeneration within 1 to 2 weeks, leaving eosinophilic "ghost" outlines of the original tumor pattern, scattered inflammatory cells with pyknotic nuclei, and some healthy fibroblasts (fig. 8). Outgrowth from tumor explants on cellophane was primarily fibroblastic, with some macrophages and giant multinucleate cells appearing in the first 1 to 3 weeks of culture.

In some histological sections of fresh or cultured tissue, one important complicating factor was noted: the presence of entrapped, normal bronchial epithelium within the tumor (fig. 7). Occasionally, ciliated epithelial cells could be seen beating in explants that had been made presumably from tumor tissue only. Sections of such pieces revealed normal epithelial cells overlying degenerating tumor (fig. 9). Epithelial outgrowth of tumor explants could not be distinguished from epithelial outgrowths of explants of normal bronchi (fig. 6).

Table 2 describes the various tumors studied and the frequency with which ciliary beating was observed in the cultures.

TABLE 2.—*Types of tumors cultured*

Tumor	Pathological finding	Ciliary beating noted
1	Squamous-cell carcinoma	−
2	Anaplastic	−
3	Squamous-cell carcinoma	+
4	Squamous-cell carcinoma	+
5	Anaplastic	−
6	Squamous-cell carcinoma	−
7	Metastatic osteosarcoma	+
8	Squamous-cell carcinoma	+

Tumor cells released from tumor pieces by trypsin and planted in gradient bottles (*2*) did not become established, but abundant fibroblastic proliferation occurred, and macrophages and giant cells persisted for 2 to 4 weeks.

Discussion

Strelin (*5*), in 1929, observed ciliary movement during 9 days of tissue culture of rabbit respiratory mucosa. Schopper (*6*), in 1935, using embryonic guinea-pig and chick-bronchial epithelium, found ciliated epithelial cells resistant to variations in temperature (25 to 28° C.), light, and poor nutrition. He noted ciliary beating up to 3 weeks of cultivation. Proetz and Pfingsten (*7*), in 1939, studied the ciliated nasal mucosa of the fetal guinea pig, in hanging drop cultures, by cinematography and histological techniques. They maintained these explants 3 to 4 weeks and noted active ciliary beating both at the edge of the

explant and in areas of outgrowth. More recent studies by Rose, Pomerat, and Danes in 1949 (*8*), on human ciliated nasal mucosa, have demonstrated retention of ciliary activity at room temperature, for 4 weeks, in phase-contrast slide cultures. With similar techniques, Danes (1949) studied pulmonary epithelium of *Triturus viridescens* and obtained active ciliary motion, with the inception of outgrowth, for as long as 6 weeks (*9*).

Hoch-Ligeti and Hobbs (*10*), in their petri-dish clot cultures on perforated cellophane, observed ciliated epithelium in their permanent strains up to the sixth "generation" (about 40 to 50 days). These strains were isolated from cultured bronchial epithelium and submucosa from adult human tissue. Barski *et al.* (*11*), using coverslips and chick-plasma clots in flattened tubes, have grown human adult and embryonic respiratory mucosa and monkey trachea as a means of testing resistance to polioviruses and adenoviruses. Ciliary activity persisted as long as 3 weeks in the human material, with isolated spots of ciliated cells in the growth area close to the fragments. The ciliary activity of the monkey trachea was evident for 45 days *in vitro*. Epithelial sheets radiated out from the explant fragments and showed mitotic figures, but ciliated cells disappeared from the growth zone between the 10th and 14th day. The illustrations in the study by Barski *et al.* show flattening of the cells lining the mucous glands after 9 days of cultivation.

While we have not succeeded in establishing cell strains from either normal bronchial or malignant lung tissue, as have Hoch-Ligeti and Hobbs (*10*), Baron and Rabson (*12*), Frisch *et al.* (*13*), and Jordan (*14*), with nasal mucosa, our results point out 2 important considerations:

1) Normal bronchial mucosa has been maintained functionally active, as evidenced by ciliary beating, for 135 days, when the experiment was voluntarily terminated. The cuboidal cells that remain as a single layer of ciliated epithelium appear to retain this state of differentiation during contact with the original connective tissue substrate. They remain differentiated only briefly when they migrate from this location.

2) The histological evidence we have obtained (fig. 7) indicates that normal bronchial epithelium may be enclosed within the tumor area. In 5 tumors that we have cultured, some ciliary beating was observed, indicating the presence of ciliated bronchial mucosa in the explant. While rapid necrosis of the tumor tissue has been the rule, normal bronchial epithelium tended to survive longer and to produce some epithelial outgrowth. The proliferating epithelial cells in the outgrowth could be maintained for several weeks and showed normal mitotic figures, but the cells were not established as a permanent strain (fig. 6).[4]

Since cells in tissue culture tend to "alter" in appearance and chromosome count upon subculture, when established as a permanent cell strain, an exact knowledge of the tissue of origin is of greatest importance. To vouchsafe that cells growing in a lung-tumor culture are of malignant

[4] After this manuscript had been submitted for publication, a cell strain was obtained which arose from a "pinhead" amount of tissue, devoid of ciliated bronchial mucosa, and to all visible appearances was from the highly differentiated mucoid adenocarcinoma tissue being cultured.

origin, the explant from which the cells grow must be known to be free of entrapped normal epithelium.

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PLATE 95

Human adult bronchial tissue. Hematoxylin and eosin.

FIGURE 1.—Fresh normal human bronchus showing pseudostratified ciliated columnar epithelium from 3 to 6 cells thick. × 275

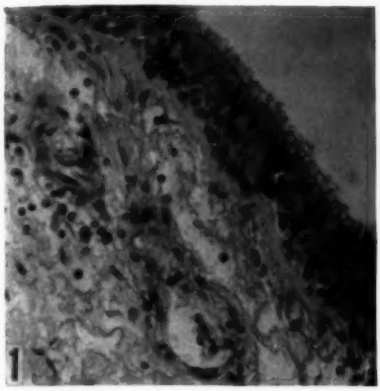
FIGURE 2.—Normal bronchus after 5 weeks' culture. Invaginated area showing good retention of columnar structure and cilia. × 275

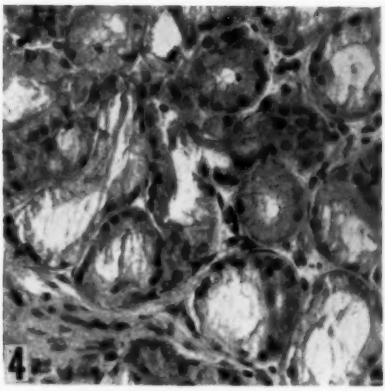
FIGURE 3.—Appearance of normal bronchial mucosa after 14 days' culture. Cilia visible in large duct (opposite *arrow*). Mucous membrane is 1 to 2 cell layers in thickness. Mucoid material is evident in a mucous gland. × 275

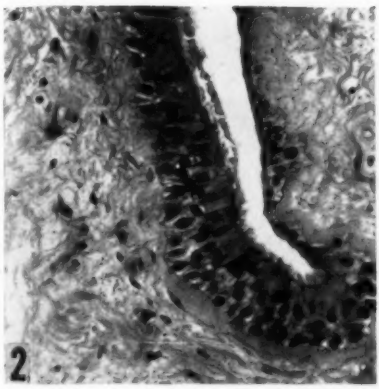
FIGURE 4.—Normal human bronchial mucous glands, fresh state. Lining epithelial cells are low columnar. Small amounts of mucus are present within lumina. × 275

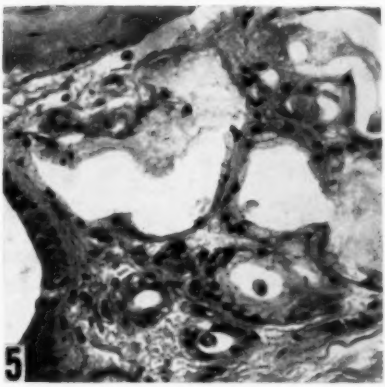
FIGURE 5.—Bronchial mucous glands cultured 1 week. Lining epithelial cells are cuboidal and somewhat flattened. Mucoid material persists in lumina. × 275

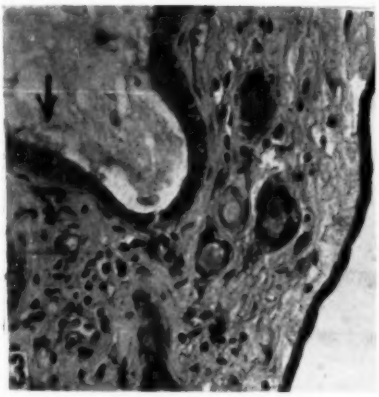
FIGURE 6.—Epithelial cell outgrowth from squamous-cell carcinoma. Grown on cellophane 3 months. Mitosis present, cells apparently normal. × 320











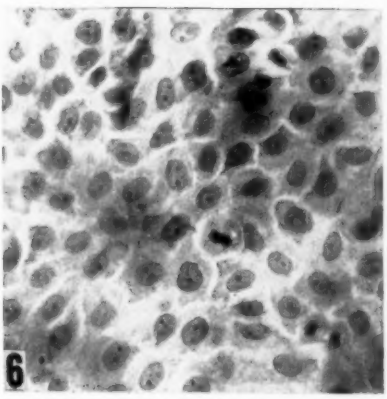


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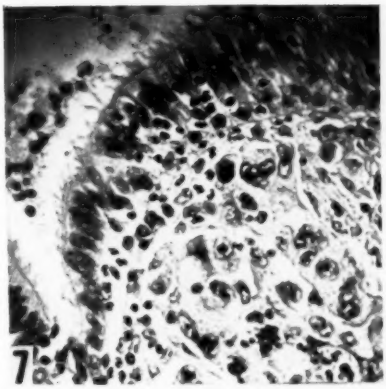
PLATE 96

Human bronchogenic carcinoma. Hematoxylin and eosin.

FIGURE 7.—Bronchogenic carcinoma, fresh state. Along one surface is non-neoplastic pseudostratified ciliated columnar epithelium. Beneath is tumor consisting of moderately differentiated squamous-epithelial tumor cells, most of which are polyhedral. × 275

FIGURE 8.—Tumor explant after 10 days in culture. Rapid degeneration of tumor cells has occurred. Some tumor cells are extensively vacuolated, others exhibit only "ghost" outlines. Scattered histiocytes with pyknotic nuclei and a few "healthy-appearing" fibrocytes are present. × 275

FIGURE 9.—Tumor explant after 10 days in culture. Tumor cells are no longer apparent. Surrounding the residual, relatively acellular fibrous matrix is a sheath of non-neoplastic ciliated epithelium. × 320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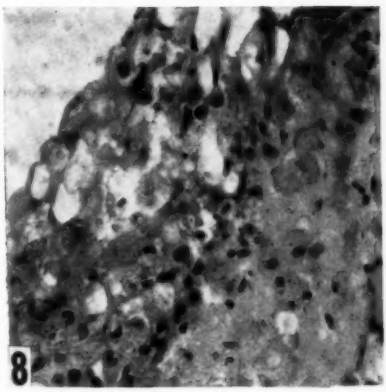
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Further Studies on Cow Anti-HeLa Serum[1,2]

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Summary

Heterologous cytotoxic activity against HeLa cells, which was produced in a cow, prevented HeLa tumors in X-irradiated cortisone-treated rats when injection was made by the same route or by a different route from that of the HeLa cells. By means of the cytotoxic metabolic inhibition test with tissue-culture cells, the cytotoxin could be traced from the peritoneum into the extracellular fluid compartment of the rat. The half life was approximately 1 day. By tumor prevention, the cytotoxin could be shown to diffuse from the blood stream back into the peritoneum. Under the conditions of the study, this heterologous cytotoxic substance, probably antibody, appears to diffuse across capillary membranes and to inhibit the gross formation of HeLa cell tumors in X-irradiated cortisone-treated rats.—**J. Nat. Cancer Inst. 22: 1039–1043, 1959.**

In a previous report (*1*), studies were presented on the lethal effect of cow anti-HeLa serum for HeLa cells, as shown by a new quantitative cytotoxic metabolic inhibition (CMI) test (*2*). The immune cow serum (ICS) injected intraperitoneally protected rats against HeLa cells that were injected intraperitoneally, and the number of cells killed per volume of immune serum was the same as in the CMI test. This close correlation was interesting and requires more explanation. Other questions raised by these experiments are: Can the CMI test detect cytotoxic activity in the blood of rats that receive the cow antiserum intraperitoneally? If so, how long does it take for the cytotoxic activity to appear in the blood stream? How long does it persist there? Can it go back into the peritoneum to attack HeLa cells that are injected subsequently? The studies presented in this paper provide answers to these questions and show that the CMI test is a sensitive measure of passively injected cytotoxins.

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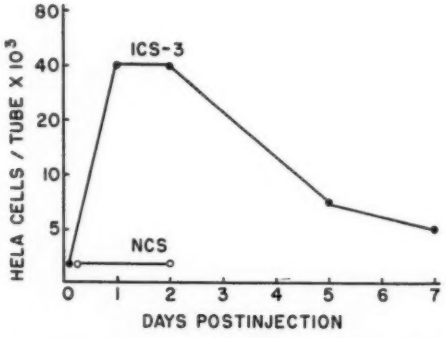
Ross (*3*) has summarized the differences between nonspecific cytotoxins and specific cytotoxic antibody and suggested criteria for the determination of specificity. The cytotoxic activity produced in the cow against HeLa cells has not been established as specific anti-HeLa antibody, though it satisfies most of Ross's criteria (*1*), and further studies on specificity will be the subject of a separate report. However, antibodies specific for HeLa cells in culture would not imply specificity directed at human cancer as such.

Materials and Methods

The cell-culture techniques used in this laboratory have been described (*1, 4, 5*). Cow anti-HeLa serum prepared for a previous investigation (*1*) was used in this study. The CMI test and the rat-HeLa tumor system in X-irradiated cortisone-treated rats were the same as in the previous report (*1*). The time interval between the injection of HeLa cells into the conditioned rats and the termination of the experiment was 21 days.

Results

The appearance of cytotoxin in the blood stream with its rate of disappearance in X-irradiated cortisone-treated rats that received intraperitoneal injections of cow anti-HeLa serum is illustrated in text-figure 1. The rats



TEXT-FIGURE 1.—The curve represents the appearance and disappearance of passively transferred cow anti-HeLa cytotoxin in the serum of X-irradiated cortisone-treated rats. Each point represents the greatest number of HeLa cells killed by 0.25 ml. of rat serum collected 1 to 7 days after the intraperitoneal injection of 3.2 ml. of immune cow serum, ICS-3 (*solid circles*), or normal cow serum, NCS (*open circles*).

did not receive HeLa cells. In these studies, 3.2 ml. of ICS-3 was injected intraperitoneally on day 0. Groups of 6 rats were then bled periodically for 1 week, and the pooled serums were tested against HeLa cells by the CMI test. The results indicate a peak of passively injected cytotoxic activity within 1 day, followed by a rather rapid fall in activity during the 1st week. The half life of this heterologous cytotoxic antibody is approximately 1 day, as shown in text-figure 1.

Prevention of Intraperitoneal Rat-HeLa Tumors by Anti-HeLa Serum Injected Intravenously

Cow anti-HeLa serum or normal cow serum was injected intravenously into the tail vein of weanling rats 2 days after they had received 250 r whole-body X radiation. After 24 hours HeLa cells were injected intraperitoneally and 5 mg. of cortisone was injected intramuscularly. The cortisone was given 3 times a week in 5 doses. The results are presented in table 1 and show that the immune serum protected against the formation of intraperitoneal HeLa cell tumors under the experimental conditions. On the basis of the CMI test *in vitro*, 3.2 ml. of ICS-3 would kill 5×10^6 HeLa cells in the test tube. In the rat-protection tests, only 1 of 17 rats receiving intravenous injections of 3.2 ml. of serum and intraperitoneal injections of 5×10^6 cells developed a small tumor. Rats receiving 1.6 ml. or 0.8 ml. of serum plus 5×10^6 cells developed more tumors, as shown in table 1. Twelve of 14 rats receiving injections of NCS and 5×10^6 cells developed intraperitoneal tumors.

TABLE 1.—*Prevention of intraperitoneal HeLa cell tumors in X-irradiated cortisone-treated rats by intravenous injection of cow anti-HeLa serum*

Serum	Serum volume (*ml.*)	HeLa cells ($\times 10^6$)	Number of rats	Tumors*			Tumors (*percent*)
				None	Small	Abundant	
Normal cow serum	3.2	5.0	14	2	3	9	85.7
Cow anti-HeLa serum (ICS-3)	3.2	5.0	17	16	1	0	5.9
	1.6	5.0	13	7	4	2	46.1
	0.8	5.0	16	4	7	5	81.2

*Tumors graded as follows: 1) abundant nodules scattered diffusely over all peritoneal surfaces with the major cluster in the omentum measuring over 10 mm. in diameter, 2) small nodules of the omentum measuring less than 10 mm. in diameter, and 3) no visible nodules.

Discussion

Previous studies by Weigle (*6*) showed that the half life of bovine serum albumin and bovine γ-globulin, following intravenous injection into 170- to 200-gm. albino rats, was 1.3 and 3 days,[6] respectively. In our previous studies, the cytotoxic activity of the immune cow serum was found to be in the γ-globulin fraction. In this report, whole cow serum was injected intravenously into weanling rats, weighing approximately 50 gm., which had received X irradiation and cortisone. The passively transferred heterologous cytotoxic activity was measured by the CMI test, and the half life of approximately 1 day is in general agreement with the previous report.

The work of some investigators (*7–11*) has shown that antitumor antibodies cross capillary membranes with difficulty and are of limited value in treating solid tumors. Others (*10, 11*) have shown that anti-Erhlich ascites tumor antibodies are effective when injected intra-

[6] Prudansky and Axelrod quoted by Weigle.

peritoneally against ascites tumor, and have speculated that this is probably because of the ready access of the antibody to the tumor cells. The present concept based on these reports is that cytotoxic antibodies do not readily cross capillary membranes. In our previously reported study (*1*) we were surprised to observe that the number of HeLa cells killed by a volume of cytotoxic antibody *in vitro* was almost the same as that tested *in vivo* in the X-irradiated cortisone-treated rat. One might expect the antibody to be more effective in the rat. Factors tending to make the serum more effective *in vivo* include: 1) damage to some cells by injection, 2) phagocytic activity of rat leukocytes, 3) lysozyme-like substances, 4) natural antibodies, 5) properdin, 6) tissue resistance to implantation, 7) sudden transfer of the cells to an environment different from their test-tube habitat in 199 medium plus 20 percent calf serum, and 8) other unknown factors. Factors tending to make the serum less effective *in vivo* are: 1) loss of serum from the rat through excretion by the kidneys, 2) diffusion of serum away from the peritoneum and inability to cross the capillary membrane for return to the intraperitoneal site of tumor cells, and 3) degradation or catabolism of antibody or nonspecific binding of antibody by rat tissues other than the tumor cells. In these studies the tumor cells were of human origin and the host was a rat. The cow anti-HeLa serum, therefore, should have contained no antibody capable of reacting specifically with rat capillary membrane. At first we thought it pure chance that the titrations, *in vitro* and *in vivo*, of anti-HeLa cytotoxins gave similar results. In the present studies, with a different route of administration of serum, the results are the same. One must conclude that, under the experimental systems used, there exists a nice balance between the factors tending to increase and those tending to decrease the effect of serum *in vivo*, with the result that titrations *in vitro* and *in vivo* are comparable. The evidence also encourages the conclusion that in the treated rat the cytotoxic antibody passes freely across capillary membranes. X irradiation of the rats is a possible explanation for the apparent discrepancy in the capillary membrane permeability to antibody, described in this paper, as compared with the work of other investigators. Wissler (*11*) has suggested the use of local X ray to increase capillary permeability and thereby facilitate immune serum therapy of solid tumors. Additional study is needed to determine whether the antibody will destroy large established tumors in the rat and the quantitative relationships involved.

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Influence of Cysteine on the Intestinal Epithelium of X-Irradiated Rats [1]

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Summary

Cysteine reduced the degree of X-ray-induced mitotic inhibition of the epithelial cells in the small intestine of rats exposed to 100 r and 300 r whole-body irradiation. For a 300 r dose level, the rate of recovery of the mitotic activity of the cysteine-treated rats was approximately the same as for the untreated rats. The time required for recovery of the mitotic activity varied with the radiation dose. A disappearance of crypt structure at the higher dose range, 1000 r and 3000 r, was noted. The protective effect of cysteine on crypt structure was reflected by increased survival in rats pretreated with cysteine at 1000 r. For 3000 r, some protection by cysteine was indicated in early mitotic-activity observations and in a slight increase in the survival time.—**J. Nat. Cancer Inst. 22: 1045–1057, 1959.**

Several hypotheses have been advanced to account for the ability of certain sulfhydryl compounds to reduce the biological damage caused by ionizing radiation (*1*, *2*). At the present time, the favored concept seems to be that these protective agents exert their action by competing for the radiation-induced products (free radicals), thus reducing the number of these radicals in the system. However, Eldjarn, Pihl, and Shapiro (*3*) recently found that when cystamine, one of the most potent protective agents (*4*, *5*), was present in a biological medium, its ability to inactivate free radicals was far too low to account for its protective ability *in vivo*. They suggested that the binding of such protective compounds as cystamine to tissue constituents was perhaps causally related to the protective ability of these compounds. More recently, Eldjarn and Pihl (*6*), using S^{35}-labeled cystamine and cysteamine compounds, reinvestigated the metabolism of these protective agents in mice. Their findings demonstrated that the major part of cystamine or cysteamine in the blood occurred in chemical combination with the blood proteins and with simpler compounds such as glutathione.

[1] Received for publication December 9, 1958.

Other investigators have attempted to localize the protective action of the sulfhydryl compounds. It has been shown that cysteine, for instance, afforded protection from many forms of radiation damage, such as mortality of mice, which was decreased (*7*), anemia and lymphopenia in mice and rats (*8*, *9*), splenic atrophy in mice (*10*), lenticular opacity in rabbits (*11*, *12*), and epilation in guinea pigs (*13*). Ransom (*14*) demonstrated that chromosome aberrations in the corneal epithelium of rats, which were caused by radiation, were reduced by cysteine. Jennings and Tessmer (*15*) have shown that glutathione reached a fairly high concentration in the spleen and liver of mice about 30 minutes after administration when the greatest radioprotective effect of glutathione was demonstrable (*16*). They concluded that the primary radioprotective effect of glutathione in total-body irradiation appeared to be in the spleen. However, Parr, O'Neill, and Korson (*17*) were unable to ascertain a protective action by cysteine on the spleen (cellular and weight changes) of X-irradiated rats. In other pertinent studies, Parr *et al.* (*18*) showed that the inhibiting effect of X rays on the nucleic acid synthesis in the intestinal mucosa of rats could be influenced by pretreatment with cysteine. They concluded that the protective action of cysteine involved such vital processes as the nucleic acid cycle. In the present report, their study is extended to an investigation of the influence of cysteine on the mitotic process in the intestinal mucosa of X-irradiated rats.

Materials and Methods

A total of 336 male Sprague-Dawley rats, weighing from 230 to 280 gm., was used. Before and after irradiation the rats were housed 4 to a cage and given Purina laboratory chow and water *ad libitum*. The nonirradiated controls were treated in a similar manner.

The rats were divided into 10 groups and treated as follows: 1) nonirradiated controls, 2) cysteine-treated controls, 3) rats exposed to 100 r, 4) rats pretreated with cysteine and exposed to 100 r, 5) rats exposed to 300 r, 6) rats pretreated with cysteine and exposed to 300 r, 7) rats exposed to 1000 r, 8) rats pretreated with cysteine and exposed to 1000 r, 9) rats exposed to 3000 r, and 10) rats pretreated with cysteine and exposed to 3000 r.

Ten minutes before irradiation, the rats received an injection intravenously, in the tail, of 2 ml. of cysteine solution (prepared with sterile water and adjusted with 2 N NaOH to *p*H 7–8) of such concentration that the rats received 850 mg. per kg. of body weight. The rats were irradiated 2 at a time in a well-ventilated lucite cage. The X rays were delivered by a General Electric Maxitron Unit operated at 250 kvp, 4.75 mm. Be inherent filtration, 1 mm. Al plus 0.5 mm. Cu added filtration, HVL 1.1 mm. Cu. The dose rate at a target-specimen distance of 50 cm. was 150 r per minute, as measured in air with a 250 r Victoreen Ionization Chamber placed in the lucite cage.

The groups of irradiated and control animals were killed by a blow to the head, at selected time intervals after irradiation. With minimum

delay, a portion of the jejunum about 2.5 cm. long was removed from the area approximately 18 cm. below the pylorus, rinsed with saline solution, and placed in Bouin's fixative. Standard procedure with hematoxylin-eosin stain was used in preparing the tissues for histological examination.

In an effort to standardize the technique of counting mitotic figures, a cell was considered to be in mitosis only between the stage in prophase at the breakdown of the nuclear membrane and the condensation of the chromosomes to the stage in telophase when the cytoplasm of the daughter cells was completely separated. The sections of the jejunum were cut 7 μ in thickness and perpendicular to the mucosa surface. Since mitoses were confined almost entirely to the epithelium of the crypts of Lieberkühn, only the areas in which these crypts appeared continuous were examined in detail histologically. The mitotic figures in 50 crypts per animal were counted in at least 4 animals, per time interval after irradiation. The results were expressed in terms of average mitotic figures per crypt.

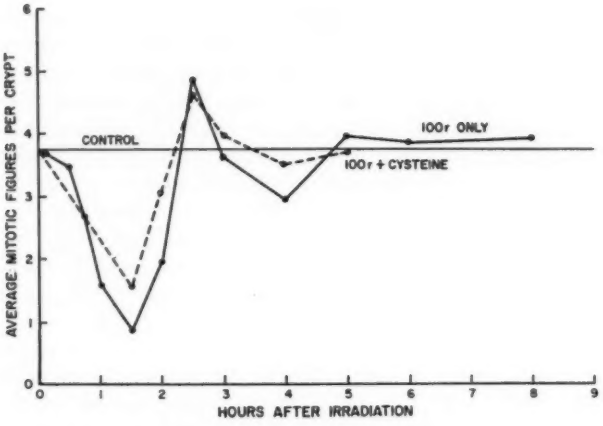
Results

The data in table 1 and text-figure 1 show the variation in mitotic activity after irradiation with a dose of 100 r, and the effect of cysteine on the X-ray-induced fluctuations. At this dose there is a trend toward lessening the variations from normal. At the 100 r level, statistical

TABLE 1.—*Mitotic activity of the intestinal epithelium following 100 r total-body X irradiation*

Hours after irradiation	Average mitotic figures per crypt		P level
	Untreated	Cysteine-pretreated	
0.17	3.70 ± 0.24		
0.5	3.48 ± 0.56		
0.75	2.69 ± 0.22		
1.0	1.57 ± 0.51		
1.5	0.84 ± 0.16	1.54 ± 0.15	.001
2.0	1.99 ± 0.50	3.05 ± 0.25	.001
2.5	4.86 ± 0.49	4.60 ± 0.46	.397
3.0	3.74 ± 0.40	3.98 ± 0.37	.30
4.0	2.93 ± 0.49	3.49 ± 0.18	.008
5.0	3.96 ± 0.25	3.74 ± 0.21	.16
6.0	3.89 ± 0.17		
8.0	3.93 ± 0.37		

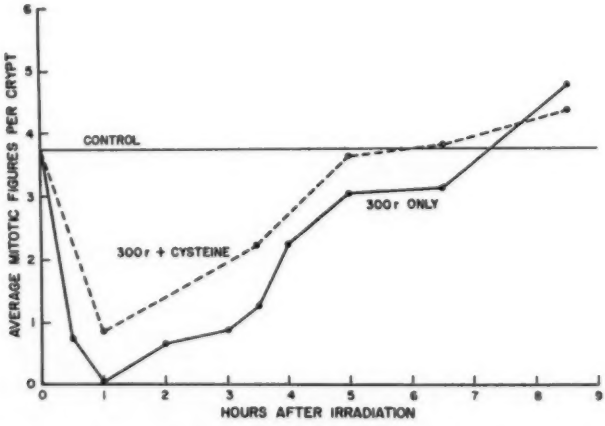
significance was noted at 1½, 2, and 4 hours after irradiation. The statistical significance of the difference between the means was determined by the standard t test. At 100 r the time of maximum depression was at 1½ hours after irradiation. The mitotic activity returned to normal between 2 and 2½ hours with a maximum rise above normal at about 2½ hours after irradiation. A secondary depression in mitotic activity was



TEXT-FIGURE 1.—Mitotic activity in intestinal crypts of cysteine-treated and untreated rats exposed to 100 r X irradiation.

observed at 4 hours. The mitotic activity returned to normal 5 hours after irradiation.

For animals irradiated with a dose of 300 r, the mitotic variations are shown in table 2 and text-figure 2. Minimum mitotic activity was noted at 1 hour after irradiation (figs. 1 through 3 show crypts of normal untreated and cysteine-treated rats at this time interval). Normal mitotic activity was reached between 6½ and 8½ hours, followed by above-normal activity. Cysteine showed a tendency to lessen the degree of mitotic inhibition at all time intervals except 8½ hours after irradiation. At this time interval there was overcompensation. Statistically significant



TEXT-FIGURE 2.—Mitotic activity in intestinal crypts of cysteine-treated and untreated rats exposed to 300 r X irradiation.

TABLE 2.—*Mitotic activity of the intestinal epithelium following 300 r total-body X irradiation*

Hours after irradiation	Average mitotic figures per crypt		*P* level
	Untreated	Cysteine-pretreated	
0.75	0.79 ± 0.13		
1.0	0.12 ± 0.17	0.88 ± 0.19	.001
2.0	0.66 ± 0.09		
3.0	0.88 ± 0.17		
3.5	1.29 ± 0.23	2.22 ± 0.31	.005
4.0	2.28 ± 0.32		
5.0	3.12 ± 0.72	3.65 ± 0.50	.192
6.5	3.10 ± 0.15	3.78 ± 0.18	.001
8.5	4.72 ± 0.53	4.35 ± 0.21	.11

differences between the mitotic activity of treated and untreated animals were noted at 1, 3½, and 6½ hours after irradiation.

In the control animals, there was no statistically significant difference between untreated and cysteine-treated animals at the time of death. Therefore, the average mitotic activity of all the nonirradiated animals was used as the control (text-figs. 1 and 2). The average for the control group was 3.75 ± 0.35 mitotic figures per crypt.

The mitotic activity of animals irradiated with 1000 r is shown in table 3. Cysteine reduced the degree of mitotic depression at all time intervals. The crypts of the untreated animals were completely or partially destroyed at 48 to 72 hours after irradiation (figs. 4 through 7). The mitotic activity in the rats pretreated with cysteine was slightly increased in comparison to untreated rats. Survival studies at 1000 r showed that 90 percent of the rats died within 4 days. The remaining 10 percent were dead by the 8th day. Pretreatment with cysteine resulted in a 5-day increase in median survival time over the untreated rats, and 25 percent were still alive at the end of 30 days.

All rats that received 3000 r and cysteine plus 3000 r were dead within 4 days. The data in table 4 show the effect of 3000 r on mitotic-activity

TABLE 3.—*Mitotic activity of the intestinal epithelium following 1000 r total-body X irradiation*

Hours after irradiation	Average mitotic figures per crypt		*P* level
	Untreated	Cysteine-pretreated	
0.5	0.24 ± 0.17		
1.0	0.05 ± 0.03	0.10 ± 0.09	.33
8.0	0.15 ± 0.05		
24.0	0.52 ± 0.18	0.78 ± 0.12	.054
48.0	Crypts absent	2.00 ± 0.08	
72.0	Crypts absent	2.50 ± 0.19	

TABLE 4.—*Mitotic activity of the intestinal epithelium following 3000 r total-body X irradiation*

Hours after irradiation	Average mitotic figures per crypt		*P* Level
	Untreated	Cysteine-pretreated	
1.0	0.05 ± 0.03	0.13 ± 0.07	.078
8.0	0.20 ± 0.10		
24.0	Crypts absent	Crypts absent	
48.0	Crypts absent	Crypts absent	

depression at 1 hour after irradiation. Within 24 to 48 hours after irradiation the crypts were destroyed in all animals.

At all radiation-dose levels, cysteine had a tendency to reduce the degree of fluctuations from the normal mitotic activity following irradiation.

Discussion

One of the first biological systems to be studied in relation to radiation damage was the small intestine. Among the earliest workers, Warren and Whipple (*19*) reported on the radiosensitivity of the small intestine in dogs. As pointed out by Leblond and Stevens (*20*), the cells in the intestinal epithelium of rats are constantly in the process of renewal, and the mitotic activity is concentrated in the crypts of Lieberkühn. The effect of X irradiation on the mitotic activity of various mouse tissues has been studied by Knowlton and Hempelmann (*21*), and the jejunum was shown to have the most rapid reaction and the earliest return to normal. Cytochemical alterations of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and alkaline phosphatase in the intestinal epithelium of the rat following total-body X irradiation were found by Tillotson and Warren (*22*). They concluded that the crypt cells of the small intestine were most sensitive and showed distinctive cytochemical modifications during the destructive and reparative phases. Finally, Quastler (*23*) asserted that intestinal radiation death in mice was linked to mitotic inhibition in the crypt cells.

The double depression of mitotic activity induced by X rays (text-fig. 1) has been observed in other biological systems. Maloy (*24*) and Knowlton and Hempelmann (*21*) explained that the primary fall was due to the inhibition of mitotic activity during or shortly after irradiation. The second depression resulted from the death of the cells which began to undergo mitosis shortly after maximum depression of mitotic activity and failed to complete the process. Thus, double depression indicated two types of cell deaths, those which died during or shortly after irradiation and those which entered into mitotic activity after maximum depression and died during division. The statistically significant difference in the mitotic activity between treated and untreated rats (table 1) at 1½ and at 4 hours after X irradiation with 100 r indicates that cysteine decreases both types of cell deaths which are caused by radiation.

While the difference between cysteine-treated and untreated rats at the time of overcompensation was not statistically significant, cysteine appeared to lessen somewhat the overcompensation in rats exposed to 100 and 300 r (text-figs. 1 and 2).

For the criterion used in this study, *i.e.*, the depression of mitotic activity in the intestinal epithelium of the rat, the findings as shown in text-figures 1 and 2 appear to support the theory that cysteine acts as a dose-reduction agent. Text-figure 2, which shows the effect of 300 r on mitotic activity and the protective action of cysteine, indicates that the rate of recovery of the cysteine-treated rats is nearly the same as for the untreated animals. There remains some controversy as to whether the protective compounds prevent cellular destruction or protect the mechanisms which accelerate regeneration. Certain organs appear to be truly protected, whereas in other organs the protector merely accelerates regeneration (*1*). It remains to be understood why some tissues act differently from others when they are irradiated under protection of various compounds. Perhaps the main reason is the ability of certain tissues to concentrate the compound, as shown by Jennings and Tessmer for glutathione and splenic tissue (*15*).

The time required for recovery of the mitotic activity of the small intestine varied with the radiation dose. Thus, in future experiments in which mitotic activity is used as a criterion, the sacrifice time following irradiation cannot be selected at random. The data for the rats exposed to 300 r (text-fig. 2) indicate that such a dose might be employed as a standard in experiments with mitotic inhibition of the intestinal epithelium as a quantitative criterion to determine the effectiveness of various physical and chemical agents. However, no specific sacrifice time interval following irradiation can be considered better than another.

The destruction of the crypts observed after 1000 r X irradiation limits the radiation dose at which mitotic activity in the intestine can be studied. However, irradiation at this dose level provides a biological effect that first appears at or near the lethal dose. It is interesting to note that the crypts did not disappear in rats pretreated with cysteine and then given a 1000 r dose. This degeneration of the crypts in mice at the 1000 r level following whole-body X irradiation has been reported by Quastler (*23*) and by Montagna and Wilson (*25*). However, this radiation syndrome was not noted by Detrick *et al.* (*26*) in rats exposed to 600 r. Thus, the appearance of crypt destruction as a radiation effect takes place at exposures between 600 r and 1000 r, which is well within the lethal range. The protective effect of cysteine on crypt structure was reflected by increased survival time observed for cysteine-treated rats given 1000 r. The increase in survival time noted here agrees with that observed by Straube and Patt (*5*) under similar conditions. Since intestinal radiation deaths normally occur in 4 to 6 days, the results indicate that cysteine either reduces or delays intestinal death. Perhaps the reduction of intestinal death is due to cysteine protection from the complete destruction of the crypts. The crypt destruction is paralleled in the findings of

Conard (*27*), who found that the greatest intestinal weight loss occurred at 2 to 3 days after an X-radiation dose of 950 r. Furthermore, Swift and Taketa (*28*), who exposed rats to 1000 r, suggested that the lack of any degree of recovery of intestinal weight on the 4th day post irradiation might be correlated with the impending death of the animal. Montagna and Wilson (*25*) stated that the cells of the villi become damaged 48 or more hours after exposure to X rays, at a time when the crypt cells are being repaired. The animals die when the villi show their greatest damage, but the crypts are largely repaired. These destructive changes in the intestinal mucosa could, at least in part, be responsible for the death of the animals.

The dose of 3000 r proved too large to provide much indication of cysteine protection. A trend toward some cysteine protection is indicated in the early mitotic-activity observations (table 4) and in a slight increase of survival time.

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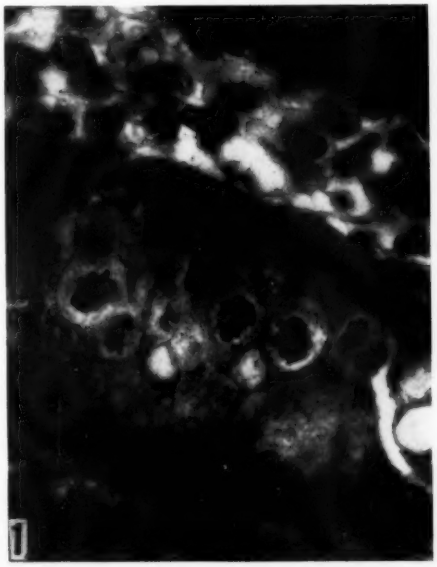
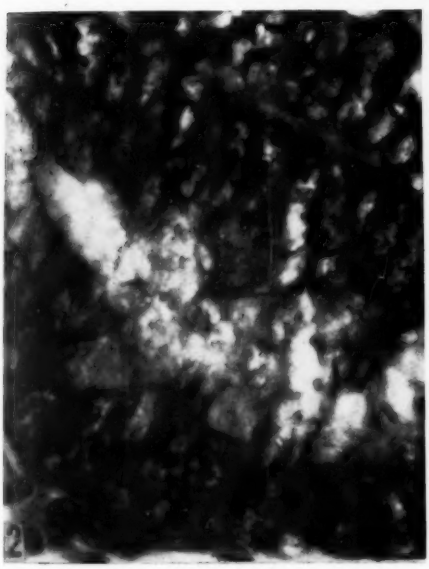
PLATE 97

FIGURE 1.—Crypts of small intestine in rat showing normal mitotic activity. × 1,400

FIGURE 2.—Crypts of small intestine in untreated rats 1 hour after receiving 300 r X radiation. × 1,400

FIGURE 3.—Crypts of small intestine in cysteine-treated rats 1 hour after receiving 300 r X radiation. × 1,400





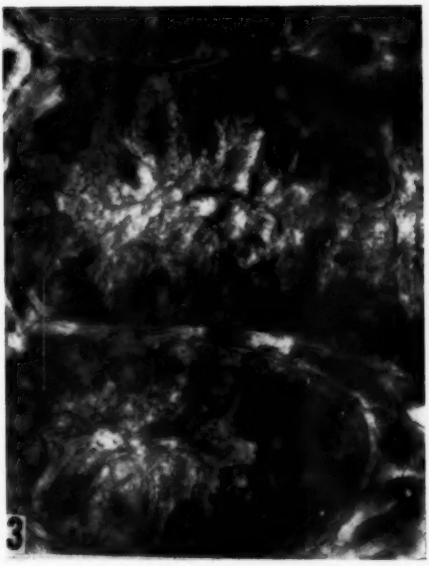


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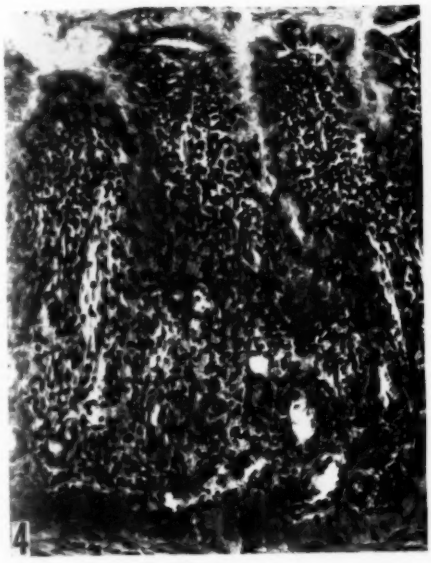
PLATE 98

FIGURE 4.—Crypts of small intestine in untreated rats 48 hours after receiving 1000 r X radiation. × 100

FIGURE 5.—Crypts of small intestine in cysteine-treated rats 48 hours after receiving 1000 r X radiation. × 100

FIGURE 6.—Crypts of small intestine in untreated rats 72 hours after receiving 1000 r X radiation. × 100

FIGURE 7.—Crypts of small intestine in cysteine-treated rats 72 hours after receiving 1000 r X radiation. × 100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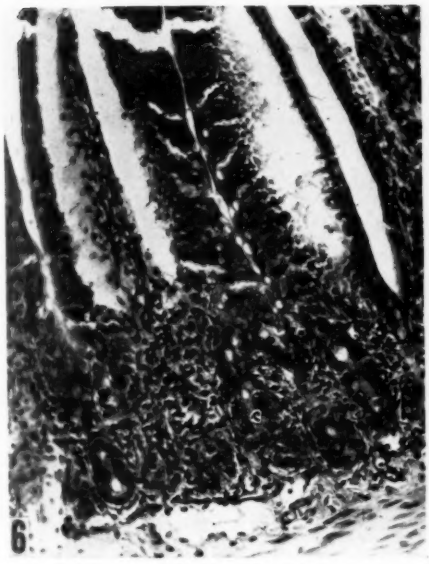
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Late Effects of Total-Body Roentgen Irradiation. V. Longevity and Incidence of Nephrosclerosis as Influenced by Partial-Body Shielding[1, 2]

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Summary

After receiving 1000 r, hypoxic, total- or partial-body irradiation, 242 female Wistar rats were observed throughout their lifespan. Hypoxic irradiation with superimposed anesthesia resulted in 67 percent, 30-day mortality. Selection of the colony by acute postirradiation deaths did not influence the magnitude of late radiation sequelae as measured by life-shortening. Growth retardation during the 2d post-irradiation year was well correlated with life-shortening. Life-shortening was observed after partial-body irradiation, to an extent approximately proportional to the weight of irradiated tissue. Nephrosclerosis was not observed unless the upper abdomen was included within the radiation field. Other than nephrosclerosis, a similar incidence of disease was observed, at death, in control and partial- or total-body irradiated rats.—**J. Nat. Cancer Inst. 22: 1059–1075, 1959.**

Exposure of the total body to sublethal quantities of externally applied ionizing X radiation has resulted in many examples of delayed or late sequelae. The tumors, cataracts, and nephrosclerosis observed in rodents after such total-body exposures have generally been ascribed to the direct action of the radiation on the individual tissues involved in these delayed pathologic changes. In the absence of a specific locus within the body that is clearly dominant in its influence on aging, the reduced longevity also observed in these total-body irradiation studies has usually been interpreted as a collective effect of radiation damage on all the vital organs of the body.

The development of some neoplasms, commonly associated with total-body irradiation of rodents, has been shown in recent years to be influenced also by complex indirect mechanisms involving other organs, in addition to the direct action of the radiation on the tissues of tumor origin. The altered endocrine balance associated with tumors of the ovary, thyroid,

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and pituitary, and the influence of nonirradiated bone marrow on the incidence of postirradiation thymic lymphoma are examples of the more systemic aspect of delayed radiation sequelae.

The *partial-body* irradiation studies reported in this paper were initially designed to study the relative importance of direct local tissue injury and of more systemic irradiation changes on the development of rodent nephrosclerosis by total-body irradiation. The reduced longevity observed in these experiments, involving radiation of less than the total body, also provides some opportunity to evaluate the contribution of various segments of the body to the shortened longevity observed in radiation-induced experimental aging.

Methods

A total of 242 young female Wistar rats, weighing approximately 180 gm., and obtained from Carworth Farms, were divided at random into 5 groups, as indicated in table 1. The 4 irradiated groups received total-body or partial-body exposure to 1000 r X radiation delivered in a single dose. The animals were anesthetized with 1.0 ml. of 1:10 intraperitoneal Myotal solution, and were in a partially protective hypoxic state from inhalation of a 5.0 percent oxygen, 95 percent nitrogen mixture, as previously described (*1*), before and during the radiation exposure. The fifth group served as nonirradiated controls and received only a comparable period of hypoxia and anesthesia.

Radiation was delivered to either the whole body or those portions of the body unprotected by the $\frac{5}{16}$-inch lead shielding, indicated at the top of table 1. Shielding of the head to the level of the clavicles excluded 11.8 percent of the body weight from the X-ray beam. Upper-abdomen shielding extending from the thirteenth rib to the lower poles of the kidneys excluded 18.1 percent of the body weight. Conversely, 81.9 percent of the total body was eliminated from the X-ray field by shielding that permitted irradiation of the upper abdomen only. Proportions of body weight protected by these lead-foil shielding procedures were determined by the mean weights of comparable frozen segments of 10 sectioned rat carcasses.

The irradiation factors were 250 kv.; 15 ma.; 0.25 mm. Cu parabolic plus 1.0 mm. Al added filters, and 0.21 mm. Cu inherent filter; 35 cm. TOD; 30 cm.^{2} field size; 1.47 mm. Cu HVL; and 185 r per minute. Two simultaneously energized targets were employed, one directed dorsally and the other ventrally. Radiation dose was measured by Victoreen thimble chambers, both in air and within the midline of 180-gm. rat carcasses. With the use of these two techniques, readings did not differ by amounts larger than the inherent error of the measuring device, which we consider to be plus or minus 5 percent.

The first group of rats was irradiated when the colony had a mean weight of 180 gm. and, based upon this weight, an estimated age of 102 days. The other 3 groups were irradiated within the following 27-day period.

After irradiation, rats were housed 4 to a cage in an air-conditioned vivarium, and fed Rockland rat diet and tap water *ad libitum*. Rats that died during the first 30 days post irradiation are also indicated in table 1. These animals were excluded from the study of late radiation effects.

Systolic blood-pressure measurements were made at sporadic and infrequent intervals during the ensuing 27-month survival period. Six rats died as a consequence of the Myotal anesthesia used during these measurements, and are also deleted from the long-term survival study. The remaining members of each group comprise those 179 rats deemed suitable for longevity statistics (table 1).

On several occasions during the 27 months of postirradiation observations, acceleration of the death rate as a result of epidemic respiratory

TABLE 1.—*Initial number of rats and selection of rats for analysis of longevity and incidence of nephrosclerosis following 1000 r, hypoxic, total-body or partial-body X-ray exposure*

Group	Irradiated: extent of lead shielding				Non-irradiated controls	Total No. rats
	Head	Upper abdomen	Entire body except upper abdomen	None		
Initial number of rats	36	40	39	79	48	242
Estimated age at radiation in days	107	102	129	109	—	—
Acute deaths during first 30 days	2	2	0	53	0	57
30-day mortality	6%	5%	0%	67%	—	—
Deaths attributed to later anesthesia	2	0	3	0	1	6
Rats suitable for longevity statistics	32	38	36	26	47	179
Autopsies performed	25	28	32	17	32	134

disease threatened to destroy the colony. At such times all surviving animals of the 5 groups received penicillin, 2000 units, and streptomycin, 0.0025 gm., subcutaneously, for 3 consecutive days during the 6th, 8th, 10th, and 13th months of the study. All surviving animals also received 1 dose of Mapharsen, 0.2 mg., and 2 doses of Chloromycetin, 50 mg. per kg., during the 13th month. The rats that received radiation only to the upper abdomen also received the 3-day course of penicillin-streptomycin, commencing on the 4th day after radiation exposure, during the period of acute radiation sickness. No other supportive care of any kind was administered.

Animals died naturally or were killed when death appeared imminent. Autopsies were performed on 134 of the 179 rats of the longevity study.

In subsequent text-figures and tables, duration of postirradiation survival is recorded to the nearest half month.

Observations

Degree of selection by 30-day mortality.—All 4 irradiated groups in this study differed from our previous postirradiation studies in that a general anesthesia was added to the conditions of radiation exposure. With this combined hypoxia and anesthesia, a low 30-day mortality ranging from 0 to 6 percent was observed after experiments that involved shielding of a portion of the body. On the other hand, under these conditions of combined hypoxia and anesthesia, total-body irradiation was followed by a 30-day mortality of 67 percent, as seen in table 1. This contrasted with an expected mortality of approximately 9 percent, which was observed in several previous studies in this laboratory when the same 1000 r, hypoxic, total-body exposure was used without anesthesia (*2*).

Decrease of longevity.—Mean and median postirradiation survival times of the 4 groups receiving either partial- or total-body irradiation were all

TABLE 2.—*Duration of postirradiation survival after partial- and total-body hypoxic irradiation with 1000 r during anesthesia**

Irradiation	1000 r				0
Lead shielding	None	Head	Upper abdomen	Entire body except upper abdomen	—
Proportion of body irradiated.....	100%	88.2%	81.9%	18.1%	0
Number rats surviving 30 days....	26	32	38	36	47
Mean postirradiation survival in months.....	12.0	13.0	12.5	17.0	20.5
Median postirradiation survival in months.....	12.0	13.5	14.5	17.5	22.0
Reduction of median postirradiation longevity (%).....	45	39	34	20	0

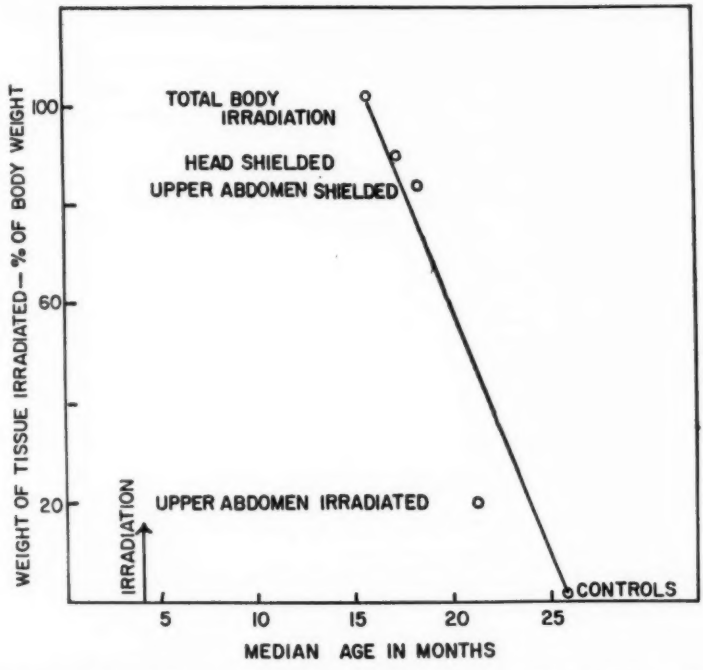
*Survival statistics are calculated after subtraction of deaths in the first 30 days.

shorter than the control survival period (table 2). Statistically significant differences of mean survival, with the use of the 5 percent significance levels of the Tukey comparison, were as follows: (100%, 88.2%, 81.9%) (88.2%, 18.1%) (18.1%, 0%). The percent figures refer to the mean proportion of the total body that was irradiated in the several groups. Any 2 groups not appearing within the same parentheses have significantly different lengths of survival. Any 2 groups appearing within the same parentheses do not have significantly different survival times.

With the use of the 95 percent protection level of the Duncan multiple range test, the following results were obtained: (100%, 88.2%, 81.9%) (18.1%) (0%). Parentheses include those groups whose survival times

do not differ significantly, as indicated.[3] It is apparent that significant life-shortening is produced by irradiation of less than the entire body.

When the duration of survival of the various groups in table 2 are equated against the proportion of the total body irradiated in the several experiments, the relationship in text-figure 1 is obtained. Reduction of lifespan possibly is directly related in some fashion to the weight of body tissue exposed to the radiation beam at this dose level. The straight



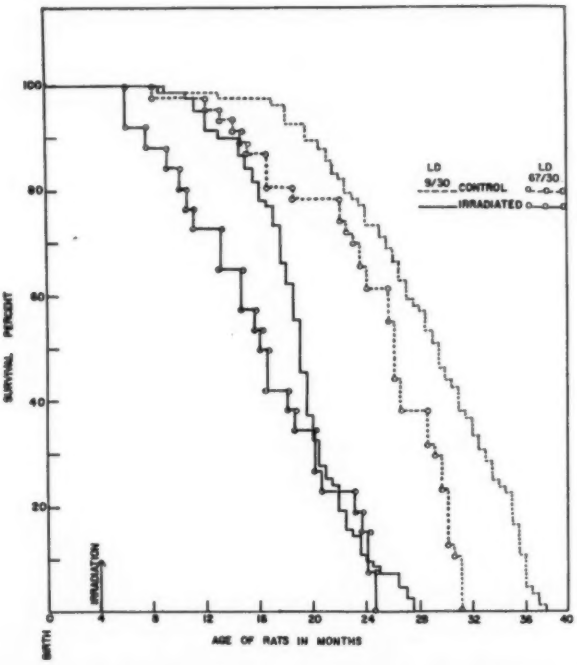
TEXT-FIGURE 1.—Median postirradiation survival following 1000 r, hypoxic, total- or partial-body irradiation. Median survival is plotted as abscissa and percent of body weight, within the radiation field, as ordinate.

line provides a reasonable fit to the few points of the present data, but this linear relationship may be only fortuitous. The reduction of longevity after irradiation of 18.1 percent of body weight of the upper abdomen is greater in proportion to the volume of tissue included in the radiation field than that observed at other points.

Selection by 30-day mortality failed to influence mean longevity of longer-term survivors.—Mean duration of postirradiation survival of the 26

[3] Tukey, J. W.: Comparing individual means in the analysis of variance. *Biometrics* 5: 99–114, 1949. Duncan, D. B.: Multiple range tests for correlated and heteroscedastic means. *Biometrics* 13: 164–176, 1957. Dixon, W. J., and Massey, F. J., Jr.: Introduction to Statistical Analysis, 2d ed., New York, McGraw Hill, 1957. We are indebted to Dr. W. J. Dixon for assistance in the statistical analysis of data.

highly selected 30-day survivors of the totally irradiated group was reduced proportionately below that of the nonirradiated controls by an amount similar to that observed in previous studies with the same radiation dose, which were not complicated by high selection from acute 30-day mortality (*2*). Present data (open circles) are compared with mortality curves from such a previous study, in text-figure 2. In the prior experiment (*2*) longevity of post-30-day survivors of a 1000 r exposure, lethal to 9.0 percent in-30 days (LD9/30), was reduced 39 percent. Mean survival following the LD67/30 total-body exposure



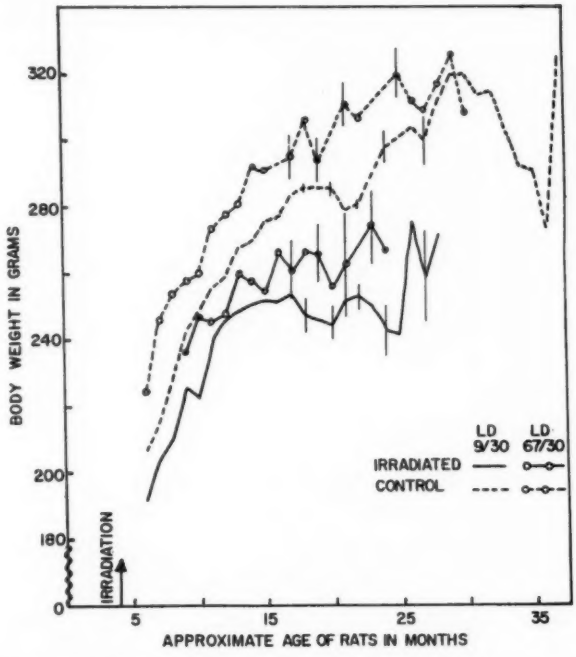
TEXT-FIGURE 2.—Postirradiation and control mortality curves of female Wistar rats after 1000 r hypoxic irradiation, with and without superimposed anesthesia. *Open* circles indicate control and postirradiation survival after 1000 r with anesthesia (LD67/30); *points* depict survival after 1000 r without anesthesia (LD9/30).

is 12.0 months as contrasted to 20.5 months in the controls (table 2). This represents a similar 41 percent reduction of lifespan.

The shorter period of survival of both irradiated and control rats in the present study (open circles) as compared to the previous experience (text-fig. 2) is probably best explained by differences of environment in the two experiments. The longer duration of life in both control and irradiated rats was observed when animals were housed in an isolated basement room, and removed from other rodents and the attendant risk of transmissible disease associated with proximity of other animals (*2*).

In contrast, the survivors in the present study were housed throughout their lifespan in a large, densely populated, air-conditioned vivarium.

Retardation of growth.—Growth curves following total-body irradiation are indicated by mean body weight of the survivors at approximately monthly intervals (text-fig. 3). Data from the present study (open circles) are again superimposed on the growth data from the 167 rats previously reported (*2*). The weight curves terminate in each group when less than 4 rats survived. Standard errors of the mean weights are



TEXT-FIGURE 3.—Postirradiation and control mean body-weight curves of female Wistar rats after 1000 r hypoxic irradiation, with and without superimposed anesthesia. *Open* circles indicate control and postirradiation mean body weights after 1000 r with anesthesia (LD67/30); *points* depict mean body weight after 1000 r without anesthesia (LD9/30).

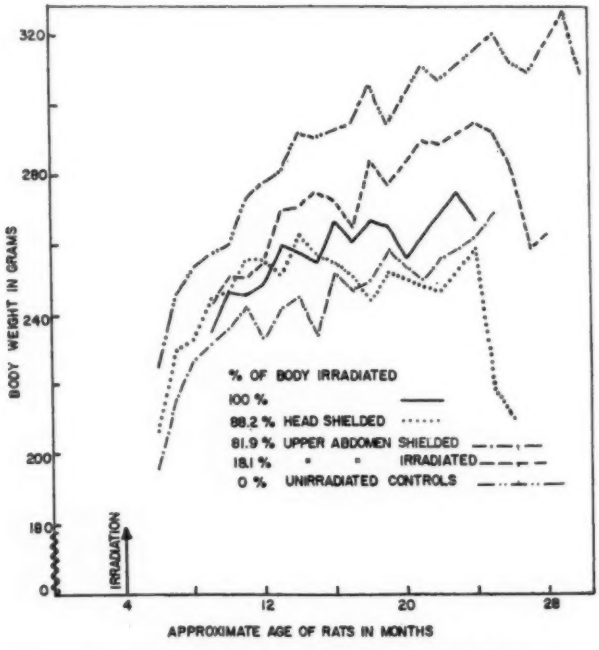
indicated at intervals during the second postirradiation year by the vertical lines above and below the mean values. Mean total weight of postirradiation survivors at 20 months, in the present study, was approximately 15 percent less than that of the controls, as contrasted to the 18 percent reduction of weight observed at 20 months in our earlier study (*2*).

A selective tendency for the larger irradiated rats of a given colony to die sooner would produce mean weight curves with this shape. Survival of the smallest and largest rats of our irradiated group was similar, however, and the suppression of mean growth during the 2d postirradiation

year cannot be explained on this basis. This retardation of growth in the irradiated group, as measured by the mean total-body weights of the survivors, accurately reflects the growth curve of the average individual rat. Increase in weight during the 2d postirradiation year was retarded. Loss of 10 to 15 precent body weight often precedes death by a few days.

It appears that the selection within the colony brought about by acute deaths occurring within 30 days of total-body irradiation, under the conditions of anesthesia and hypoxia of the present study, did not alter the composition of the colony as measured by the effects of radiation upon either the process of aging or the capacity for maximum growth. Data from both the present and earlier experiments further suggest that there is an association between the process of aging and the capacity to produce new tissue as measured by increase in body weight.

Longevity and growth in partially irradiated rats.—In general, a rough correlation between longevity and capacity to grow also appeared in the partially irradiated groups, as revealed in the mean body-weight curves in text-figure 4. The rats with only the upper abdomen irradiated, representing 18.1 percent of the body weight, had the least degree of reduction of median longevity, 20 percent (table 2), and the smallest retardation of growth at 20 months (approximately 7%). The totally irradiated group was 15 percent smaller than the controls at 20 months.



TEXT-FIGURE 4.—Postirradiation and control mean body-weight curves of female Wistar rats after 1000 r hypoxic total- or partial-body irradiation.

In the other two experiments in which 81.9 and 88.2 percent of the body was irradiated, weight curves were too similar to the weight curve of the totally irradiated rats for separate interpretation.

Autopsy Findings

Complete autopsies were performed on 134 of the 179 rats of the long-term study. With the exception of nephrosclerosis, all groups showed a similar spectrum of disease. Acute inflammations of the lung, pleura, or pericardium were the principal causes of death in the majority of rats of each group. Occasionally, peritonitis, acute pyelonephritis, acute otitis media, and purulent distentions of the fallopian tubes were also encountered. The malignant and the benign tumors observed are tabulated in table 4 and are separately discussed later. Many of the older rats also experienced epilation of the head, neck, and dorsum of the trunk. The extent and locus of this epilation bore no relationship to the region of the body within the radiation field.

Prevalence of nephrosclerosis.—The incidence of nephrosclerosis in the various experiments is shown in table 3. A high degree of characteristic nephrosclerosis (*3*, *4*) was observed in all 3 groups when the upper abdomen and kidneys were within the radiation field.

The bulk of the liver and spleen, all of the kidneys and adrenals, many loops of bowel, and a portion of bone marrow were protected from radiation by shielding of the upper abdomen. This shielding procedure eliminated the type of nephrosclerosis characteristic of total-body irradiation in our Wistar rats (*3*, *4*). This absence of radiation-induced renal disease is in contrast to the 56 percent incidence of nephrosclerosis in the head-shielded study and 35 percent incidence in the totally irradiated group. When only the upper abdomen, including the renal area, was irradiated, 22 percent developed nephrosclerosis. In the latter experiment, when 81.9 percent of the body was protected from irradiation, the 22 percent incidence of nephrosclerosis was not significantly less than that of other

TABLE 3.—*Incidence of nephrosclerosis in partially shielded female Wistar rats compared to rats surviving 1000 r, hypoxic, total-body irradiation*

Extent of body shielding	Months postirradiation survival:					Total
	2 to 6	7 to 12	13 to 18	19 to 24	25 to 30	
	N* D*	N* D*	N* D*	N* D*	N* D*	
Head	0 3	1 3	8 12	5 7	— —	14(56%) 25
Upper abdomen	0 2	0 5	0 12	0 9	— —	0 28
Entire body except upper abdomen	0 1	0 6	1 9	6 16	— —	7(22%) 32
None	0 0	2 6	4 6	0 5	— —	6(35%) 17
Controls—no irradiation	0 1	0 1	0 2	1 12	0 16	1(3%) 32

*N=rats with nephrosclerosis; D=total deaths.

groups which also received radiation to the upper abdomen as part of more extensive exposures. The renal disease did, however, occur later than in the other two experiments. Animals of this upper-abdomen radiation study were also approximately 3 weeks older at the time of irradiation than the head-shielded and unshielded groups (table 1).

Blood-pressure measurements were too infrequent in this study to provide useful data.

Nephrosclerosis as a cause of death.—The majority of rats with severe nephrosclerosis died of pneumonia. In the absence of chemical evidence of nitrogenous retention, the extent that renal failure may also contribute to death is uncertain. From the histologic appearance of the more severely damaged kidneys, renal failure would be anticipated. The majority of the glomeruli were severely sclerosed, with complete obliteration of the capillary tufts (*3*).

On the other hand, the incidence of nephrosclerosis did not correlate well with the median or mean survival of the several groups of rats. For example, in the head-shielded study, nephrosclerosis was found in 14 of 25 rats. When the upper abdomen alone was shielded, the incidence of nephrosclerosis was zero, yet longevity was increased very little (table 4).

TABLE 4.—*Incidence of nephrosclerosis and postirradiation survival after 1000 r, hypoxic, total- or partial-body irradiation*

Percent of body irradiated	Portion shielded	Incidence of nephrosclerosis	Median survival (*months*)	Mean survival (*months*)
100	None	6/17	12.0	12.0
88.2	Head	14/25	13.5	13.0
81.9	Upper abdomen	0/28	14.5	12.5
18.1	Entire body except upper abdomen	7/32	17.5	17.0
0	None—nonirradiated controls	1/31	22.0	20.5

The increase in median survival was roughly proportional to the increased weight of tissue protected from irradiation by the increase in shielding from 11.8 to 18.1 percent of the total-body weight.

When the upper abdomen alone was irradiated, median survival time was reduced to 17.5 months from 22.0 months in the controls. This reduction of lifespan is larger than would be expected from the proportional weight of tissue irradiated, as can be seen from the plot in text-figure 1. The delayed nephrosclerosis in this upper-abdomen irradiated group cannot explain the reduced longevity as measured by median survival, for only 1 of the 16 rats that died during the first 18 months post irradiation developed this lesion (table 3). Nephrosclerosis was nearly absent during the 17.5-month period when 50 percent of the upper-abdomen irradiated rats died. Irradiation-induced malfunction of the kidneys or some other

organ located in the upper abdomen, such as the adrenal, may still be specifically related to shortened longevity without histologic evidence of this disturbance.

Incidence of Neoplasms

The specific tumors encountered in the various groups of rats are tabulated in table 5. The time distribution of the malignant tumors, at

TABLE 5.—*Tissue distribution of benign and malignant tumors following 1000 r, partial- and total-body irradiation*

Tumors	Extent of shielding:				
	Head	Upper abdomen	Entire body except upper abdomen	None	Control—none
Breast—adenoma	8	10	6	9	4
carcinoma		2			
Ovary—benign	6	2	1	4	1
malignant					1
Lung—benign		1	1		
malignant					1
Lymphoma					1
Disseminated	1	2	2		2
Pituitary		2	2		2
Stomach—malignant					1
Pancreas—islet cell			2	1	
Liver—hemangioma	1	1	2	1	
Kidney—benign	1	1	2		
malignant			1		
Heart—benign	1				
Peritoneum—malignant		1	1		
Soft-tissue sarcoma	1				
Adrenal adenoma	1				
Pudendal gland—adenoma	1	2			
carcinoma		1			
Skin of jaw—carcinoma		1		1	
Uterus—malignant		1			
Bladder—cystadenoma		1			
Salivary gland—adenoma				1	
Number of malignant tumors	2	8	4	1	6
Number of benign tumors	19	20	16	16	7
Number of rats	25	28	32	17	32

TABLE 6.—*Incidence of malignant tumors at autopsy during various periods of postirradiation survival following 1000 r, hypoxic, total- or partial-body irradiation*

Extent of body shielding	Months postirradiation survival								Total	
	7 to 12		13 to 18		19 to 24		25 to 30			
	Tumors	Deaths	Tumors	Deaths	Tumors	Deaths	Tumors	Deaths	Tumors	Deaths
Upper abdomen	1	5	3	12	4	9	—	—	8	26
Head	0	3	1	12	1	7	—	—	2	22
Entire body except upper abdomen	0	6	1	9	3	16	—	—	4	31
None	0	6	0	6	1	5	—	—	1	17
Controls—no irradiation	0	1	0	2	2	12	4	16	6	31

death, is further indicated in table 6 where the incidence of malignancy is tabulated according to 6-month survival periods. In our previous studies the total incidence of malignancy in the irradiated female rats of this Wistar strain did not exceed that observed in the controls when the latter were allowed to live to old age (*2*). In this smaller number of animals of the present series the high tumor incidence in the controls that lived more than 24 months post irradiation was again apparent.

Our previous experience with neoplasms in this strain of female Wistar rats indicates that a wide variety of tumors may be expected in old rats. Irradiation, in our studies, has probably not specifically increased the incidence of any given tumor type, with the exception of ovarian tumors (*2, 3*). The present data are consistent with this generalization.

In these studies, the choice of areas of the body to be protected from irradiation by lead shielding was made with the intention of observing primarily the effects of selective partial shielding on the incidence of nephrosclerosis. Limited information regarding the location of malignant neoplasms with reference to the radiation field can also be obtained. The upper-abdomen irradiated and upper-abdomen shielded groups offer the best opportunity for comparison. When the entire body, with the exception of the upper abdomen, was irradiated (81.9% of total body weight), 5 malignant tumors were observed within the irradiation field. The other 3 malignant tumors observed in this group were either widely disseminated, with primary site uncertain, or were located too close to the margin of the shielding to be placed with certainty either within or without the radiation field. When only the upper abdomen received radiation (18.1% of body weight), 1 kidney malignancy was primary within the radiation field, but the location of the other 3 malignant tumors was indeterminant for the same reasons as given earlier. In the irradiated rats in this study, no malignant tumor was observed in tissues that were definitely shielded during the irradiation exposure.

Discussion

Longevity, as we observe it in human beings and in the laboratory animal, is influenced by the lethal effects of specific diseases and the consequences of aging. To separate aging from all disease states is a difficult matter. Processes that were considered as inevitable not many years ago and normal consequences of advanced years such as arteriosclerosis are now viewed as the result of suboptimal environment or abnormal physiology. In practice, when we have eliminated all *known* effects of specific disease, those tissue changes that are left in association with advanced age are referred to as the changes of aging.

In human pathology it is often easy to recognize death as the result of some clearly morbid process. However, in people who are 90 or 100 years old, the absence of marked chronic disease of all organ systems is often the conspicuous finding. This relative freedom from severe chronic disease in some elderly people no doubt, in part, explains their occasional long survival. Death under such circumstances is usually effected by

bronchopneumonia as a terminal illness. The ability to resist such terminal infections is obviously an important characteristic of the person with the capacity for long life.

In the nonirradiated female Wistar rat, as observed in this laboratory, some of our control groups have had a mean survival of 28 months, with many rats living from 36 to 40 months. Even at this advanced age for the laboratory rat, the plateau in the mean weight curve has not been reached. Most terminal illnesses in these old rats are readily explained by acute inflammations, presumably of infectious origin, involving vital organs, such as the lungs, pericardium, or peritoneum. When death occurs, most often from pneumonia, the cardiovascular renal system, by microscopic examination, appears to have ample reserve. Our rats have been remarkably free of severe, generalized, so-called "degenerative" changes of the circulatory system that have so complicated the distinction between disease and aging in human beings. We have not observed significant arteriosclerosis of large or middle-sized vessels, and we rarely have observed inflammatory vascular disease such as periarteritis or generalized arteriolar disease in our control animals.

In contrast, in older nonirradiated control animals, both benign and malignant tumors commonly create conditions not compatible with life. When these tumors are excluded, longevity in our control rats appears to be largely a matter of resistance to infection. If one assumes that this resistance to infection is related in some obscure manner to the age of the animal, then our longevity statistics also bear on the process of aging.

The pattern of disease in the *postirradiated* rat was remarkably similar to that observed in the nonirradiated controls. Inflammatory diseases of the thoracic organs and benign and malignant tumors dominated. In addition, occasionally a severe renal disease involving primarily the glomeruli and renal vessels of small caliber was superimposed. This renal disease was related in some fashion to irradiation-stimulated hypertension (*4*), and was distinct from the fibrotic contracted kidneys that resulted from direct renal exposure to much higher radiation doses (*4*, *5*).

Inclusion of the kidneys within the irradiation field has so far been necessary in these studies to produce this hypertension-associated nephrosclerosis in the irradiated animal. We have not yet, however, produced this lesion in irradiated rats without the concomitant existence of hypertension, when adequate prior blood-pressure data have been available. Also, the hypertension appeared to antedate the development of nephrosclerosis (*4*). Unfortunately, the blood-pressure determinations in the present study were too sporadic to provide additional information on this subject.

Rats of the present study developed nephrosclerosis after irradiation of the upper abdomen alone, but failed to do so when the upper abdomen was protected from irradiation by lead shielding. Other organs than the kidney were involved in these upper-abdomen radiation-shielding studies. We have, for example, not yet excluded the adrenal as the organ in the upper abdomen responsible for postirradiation hypertension. Studies

in progress in which we have selectively irradiated only the adrenals or kidneys following operative exposure of these organs are not yet complete. Possibly irradiation of the kidneys is necessary to produce the hypertensive state that is a prerequisite for the development of the histologically evident renal disease, which follows radiation at this 1000 r, hypoxic dose level.

The peak of the animal's capacity to increase in size is also reached in these postirradiated rats in contrast to the control group whose mean body weight increases throughout the major portion of the lifespan. The important characteristic of shortened longevity, in the postirradiated, female Wistar rats of this report, with its apparent relationship to a decreased resistance to infection, appears also to be closely associated with a decline in net capacity for accumulation of new tissue. Studies of growth based on skeletal structure have not yet been attempted.

Existing data pertaining to the general late effects of low or moderate doses of radiation of less than the total body are particularly meager. Studies by Kaplan (*6*) and Lorenz (*7*) of the effect of partial-body irradiation in C57BL mice were directed specifically toward the incidence of radiation-induced lymphoma rather than the more general problem of the late-effects syndrome. Wilson's study of upper abdominal irradiation in rats distinguishes between postirradiation hypertension and direct radiation-induced renal fibrosis (*5*). The work of Maisin *et al.* (*8*) provides the bulk of the published life-shortening data on the rat. Kallman and Kohn have also demonstrated a life-shortening effect of partial-body irradiation in the mouse (*9*).

Following 600 r total-body irradiation (LD50/30) Maisin *et al.* (*8*) observed a post-30-day, 50 percent survival of 10.8 months in contrast to the 18 months of the controls. In their studies, shielding an 8.0 $cm.^2$ region of the chest (LD2.5/30) or midabdomen (LD12.5/30) failed to appreciably lengthen the lifespan of the post-30-day survivors, although admittedly the proportions of the body shielded were small.

Similar partial-shielding studies following 850 r, in Maisin's report (*8*), cannot be compared to total-body irradiation because of absence of long-term survivors of the 850 r total-body exposure. Nevertheless, in experiments in which the total body, except for the abdomen, was shielded from the 850 r dose, longevity was reduced substantially from that observed in the nonirradiated controls. Our data support the findings of Maisin, indicating that irradiation to the abdomen alone is followed by reduction of lifespan in the post-30-day survivors. Maisin suggested that this shortened longevity was related to diseases of the abdominal organs, but autopsy details are lacking.

When the total body is irradiated, if the injurious effects are in part irremediable, it would be logical to assume that each of the individual tissues would be the seat of residual ionization-produced damage. The sum of these damages in all tissues could be injurious to the total organism by impairment of the individual specific functions which are unique to the vital organ concerned. It is also conceivable, since there are no auton-

omous organs in the body, that ionization tissue damage also has a less specific aspect relating rather more to some common denominator present in all tissues yet essential and available to the entire integrated animal body. In the latter case the summation effects of total-body irradiation damage would be less organ-dependent and more likely related to total weight of all tissues included within the radiation field.

If regions of the body containing vital organs, such as the brain, heart, or liver, were exposed to radiation doses sufficiently large to cause tissue damage as the result of delayed vascular injury, the influence of local irradiation upon longevity might be a combination of both organ specific and nonspecific mechanisms. The 1000 r hypoxic radiation used in these studies is equivalent to approximately 425 to 500 r delivered in normal oxygen tension in its effects on longevity (*2*). Doses of this relatively small size have not caused a generalized severe sclerosis of vessels, though the renal lesion observed in these studies in association with hypertension (*4*) is possibly related to clandestine renal vascular injury. The unilateral renal damage in rats, reported by Maisin (*8*), following a dose of 850 r in air, suggests that our hypoxic 1000 r may be only slightly below the threshold for histologically demonstrable direct renal radiation injury.

Nevertheless, when only a portion of the body was irradiated with 1000 r administered during hypoxia, we also observed reduced longevity, although of lesser degree than that following total-body irradiation. This, in part, may possibly be explained by the accelerated appearance of malignant tumors within the irradiated area. In upper abdominal irradiation, it may be explained, in part, in some cases, by hypertension-related nephrosclerosis, though in this study the nephrosclerosis observed did not appear sufficiently soon to affect appreciably the median survival figure. But the lifespan of rats that received partial-body irradiation and were free of both tumors and nephrosclerosis was also shortened. Their terminal illnesses were similar to those of the totally irradiated rats and the controls, and most of them died of pneumonia. Possibly we are concerned here with a submicroscopic injury to some organ such as the adrenal or a nonspecialized type of universal tissue damage of the type mentioned in our preceding speculations.

Consequently, we have searched for a possible relationship between the reduction in lifespan in our partial-shielding experiments and the proportion by weight of the total body receiving the ionization challenge. It may be fortuitous that shielding the head, or the upper abdomen, or 81.9 percent of the total body increases longevity by degrees roughly proportional to the weights of these regions. The increased longevity may be related only to more normal specialized function of these regions, all of which contain organs vital to survival. It is also possible that the sum total of all ion pairs formed by radiation absorption in all parts of the body, regardless of locus, is one critical factor bearing on aging. We do know that animals receiving upper-abdomen irradiation do not all die of demonstrable upper abdominal disease as determined by standard autopsy techniques. The proof of the existence of a form of late radiation damage

capable of influencing longevity but not related to known functions of specific organs obviously will depend upon more data. Longevity following the selective irradiation of portions of the body which clearly contain no organs known to be vital to survival will be of interest. Such studies are in progress.

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Growth of Mouse Ascites Tumor Cells in Diffusion Chambers. II. Lysis and Growth Inhibition by Diffusible Isoantibody [1, 2]

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Summary

Isoantibody was injected into mice in which the DBA/2 lymphoma was growing rapidly inside sampling-type diffusion chambers. Many of the tumor cells were lysed within 4 hours, some recovery was evident by 24 hours, and the cell count inside the chamber increased rapidly within 48 hours. Lysis was roughly proportional to the amount of serum injected. Recovery of the tumor-cell population could be delayed, apparently indefinitely, by repeated injections of antiserum. Lysis or growth inhibition could be shown for several other ascites tumors including an Ehrlich clone and MC1M sarcoma. Two lymphomas, although lysed at high serum doses, appeared to have an accelerated rate of growth after injection of small volumes of antibody. When this acceleration was studied in greater detail, it was found that the rise in cell count followed a slight initial fall. Reasons for the failure of antibody to lyse all the tumor cells are not fully understood since the population may be completely destroyed in tests *in vitro;* the availability of complement appears to be a limiting factor.—J. Nat. Cancer Inst. 22: 1077-1092, 1959.

In an earlier paper it was shown that ascites tumor cells grow freely in diffusion chambers, and their growth rates can be plotted (*1*). Isoantibodies (prepared in one mouse strain against tissues—usually neoplastic—from a genetically dissimilar mouse strain) can pass through the Millipore walls of the chambers to bring the antibody concentration to approximately that found in the abdominal cavity. Some of the results obtained, when antibody is injected into mice that have chambers containing actively growing populations of ascites tumor cells, are presented in this paper.

Experimental Procedures

A counted suspension of freshly collected ascites tumor cells was introduced through the access hole of a sampling chamber. The chamber

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consisted of a lucite cylinder, 5 mm. high and 16 mm. in diameter, closed at top and bottom by a disc of HA Millipore filter sealed to the cylinder with Acriloid® cement. An access hole admitting a 23-gauge needle was drilled in the cylinder wall. After the chamber was filled, the hole was closed by a tapered lucite peg cemented in place with glue made by dissolving a Millipore filter in acetone. The filled chamber was placed in the abdominal cavity of an anesthetized mouse. In most experiments between 12 and 20 chambers were used; usually half were for controls, but for some tumors, when the slope of the growth curve was well established, only sufficient chambers to ensure that the experiment was conforming to the normal pattern were employed for this purpose. The experimental animals received an intraperitoneal injection of antibody at predetermined intervals, the chambers were removed, and the remaining cells counted. A detailed description of the materials, presenting a number of growth curves and data on the concentrations of antibody reached inside the chamber, has been published (*1*).

The technical procedures involved are time consuming and limit the size of each experiment. For 1 person working alone, 12 chambers have been found to be the usual daily limit; for 2 people, about 20.

The greatest care was taken with collecting and counting the cells to reduce sampling errors. Most of the experiments were repeated at least 2 or 3 times. The results presented in the text-figures are all from typical experiments unless stated otherwise.

The growth curves for each tumor usually agreed closely with the average curves already established. There were, however, variations from them, especially in the extent of the lag before the phase of rapid growth began; occasionally there was a slower or more rapid rate of the growth phase itself. An unexpected complication was met in some antibody-treated chambers. The clot was sometimes considerably more dense and appeared to be of a greater mass than in the corresponding control. These clots especially were carefully triturated and sometimes gently homogenized to free the cells bound in the clot.

It is difficult to work on any quantitative basis in actively immunized animals because the total content of the antibody pool is unknown. A few experiments were in fact performed in which chambers were inserted into actively immunized hosts, and in which the fall in cell count persisted until there were very few cells remaining inside the chambers. On the other hand, the experiments were not uniformly successful and a number of them gave results strikingly similar to those obtained after passive immunization. We hope to present the question of active immunity in a further series to be reported separately.

The following experiments were performed in passively immunized mice, usually of the resistant genotype in which the antibody was originally made. All the experiments were performed when the tumor cells were in their most active growth phase, either immediately after the fall in cell count or at various stages of the near logarithmic phase of growth.

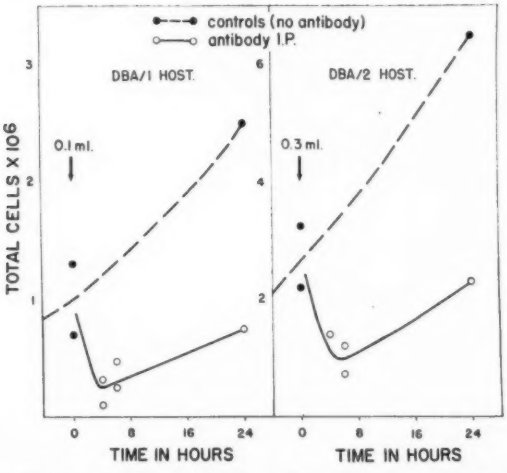
Fresh mouse isoantibody by itself shows little cytotoxic effect *in vitro*.

Since there was some doubt as to the effectiveness of mouse complement to mediate lysis, the first group of experiments was designed to see whether the antibody was cytotoxic or merely inhibitory *in vivo*, and whether cells in the later stages of growth were more resistant to antibody—established tumors are notoriously resistant to transferred antibody.

Results

Inhibitory Effect of Single Injections of Antibody

Numerous experiments were performed in which an ascites tumor was allowed to grow for a predetermined time, or until the cell concentration reached a desired level, when the host was injected with the appropriate antibody. Control animals usually received no injection, though a number of controls were given normal mouse serum with no apparent effect on the chamber population. The most extensively used tumor was the DBA/2 lymphoma because it is reasonably sensitive to antibody, grows rapidly in the chambers, and shows no marked reduction in growth rate until cell concentrations in excess of 20 million are reached. Most experiments were performed with a population of less than 10 million so that reduction of growth rate from an overcrowded population was unlikely to occur.



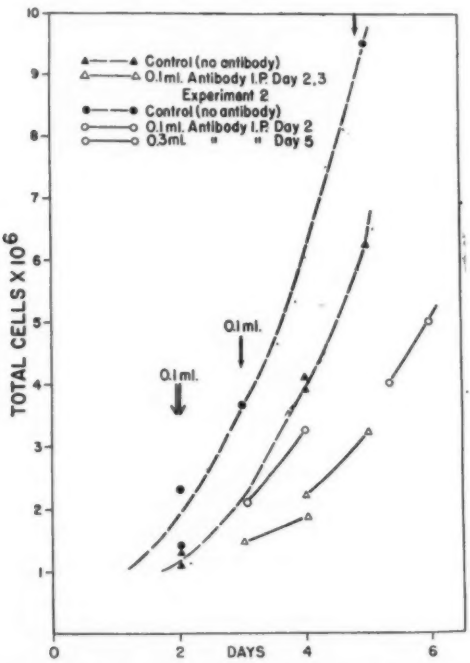
TEXT-FIGURE 1.—The immediate lytic effect of isoantibody on the DBA/2 lymphoma in its early phase of rapid growth in native or foreign hosts.

Typically, as shown in text-figure 1, the tumor was grown in DBA/1 hosts, and the antibody DBA/1-anti-DBA/2 thymoma was injected at a fairly early stage in cell growth. There was as much as 75 percent lysis of the population after a single injection of antibody, indicating that there was a cytolytic effect of the isoantibody *in situ* and also suggesting that whatever the deficiencies of mouse complement in the test tube, it was quite effective in the animal.

Concomitant studies were performed in which the cells were placed in a 1/2000 solution of trypan blue in Ringer's solution. Dead or damaged cells stain blue, superficially undamaged cells remain unstained. The frequency of stained cells is usually higher in a chamber population than in a free ascites in the corresponding growth phase. The chamber cells tend to fluctuate between 6 and 20 percent stained, but after antibody treatment the proportion of stained cells in the unlysed residue is generally about twice that of the controls and may reach considerably higher values. The chambers were usually relatively free from cell debris, even after lysis of several million cells.

In one exceptional experiment with the DBA/2 thymoma, there was an increase in cell count over that of the controls at 4 hours, with no increase in damaged cells. After 8 hours, the increase was still marked, but 61 percent of the cells now stained with trypan blue, and after 24 hours, the total number of cells had fallen to about 25 percent of the control value taken at the same time.

The general indication from these experiments is that cell death is rapid, and that lysis or autolysis is as complete as in the experiments *in vitro* with guinea-pig complement (*2*). As indicated in text-figure 1 and from other experiments (not shown) in which samples were taken at

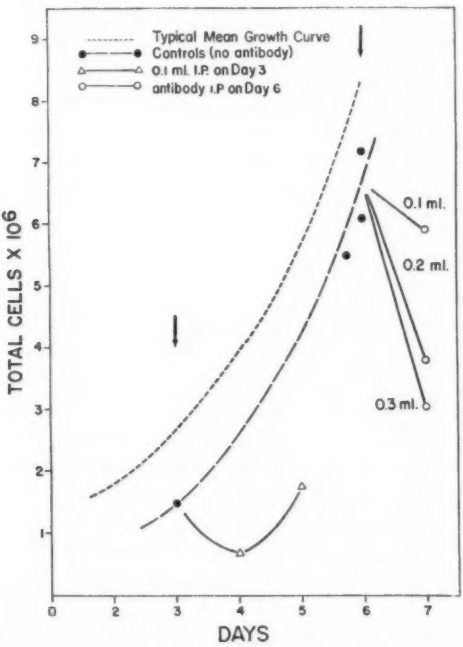


TEXT-FIGURE 2.—The effect of isoantibody at different stages in the rapid growth phase of the DBA/2 lymphoma. Samples taken 24 and 48 hours after single injection of antibody.

8 and 12 hours, the decline tends to be maximal within 4 hours after injection, and by 12 hours no further destruction occurs. In the majority of experiments, the cell concentration then begins to increase, and by 48 hours, growth rate parallels that of the controls (text-fig. 2). The results are essentially similar whether the serum injection is given early or late during the optimal growth of the tumor.

A fall in cell count was observed whether an isologous or homologous host was used, but larger serum doses were necessary in the isologous host to achieve the same result. Rapid absorption of *H-2* antibody by the isologous host is known to occur, and when the chamber fluid was titrated, no hemagglutinins were detected. This might suggest that "X" type antibodies (*3*) are effective in this system, but it is possible that *H-2* antibodies do enter the chamber soon after the injection and are immediately absorbed by the tumor cells. The question of the relative efficiency of *H-2* and "X" type antibodies could, to some extent, be resolved by preadsorption with isologous tissues, but falls outside the scope of the present investigation.

In one of the experiments shown in text-figure 2, a somewhat larger serum dose was used for the later injection with possibly a greater effect than that obtained with 0.1 ml. This effect was studied in more detail in a series of dose experiments, one of which is illustrated in text-figure 3.



TEXT-FIGURE 3.—Comparison of the effect of different volumes of isoantibody on the chamber populations of DBA/2 lymphoma grown in DBA/1 mice.

Augmentation of Effect by Increasing the Antibody Dose

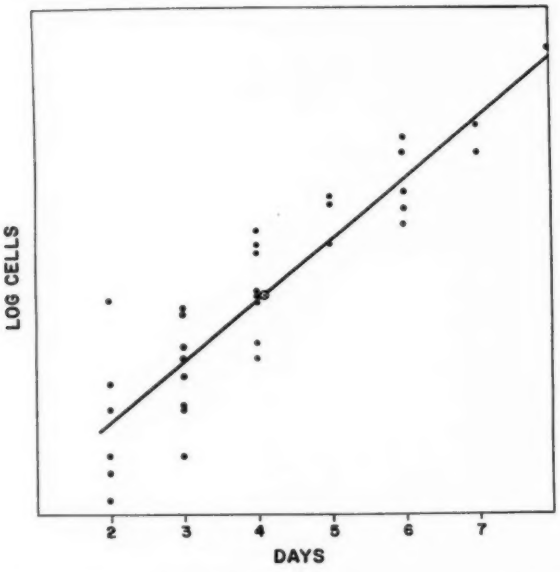
Each of the determinations shown in text-figure 3 follows a single injection of antibody 24 hours earlier. It will be noted that 2 control curves are presented in this and in several other figures. The finely stippled line represents the "typical mean growth curve" obtained by plotting 33 control values from the 2d to the 8th day for 7 separate experiments (text-fig. 4), and has been drawn in wherever pertinent to indicate the probable cell concentrations at intermediate points where no direct controls are available.

Injections were made either in the early stages of growth or when the population had reached about 6 million cells. Earlier injections of 0.1 ml. (only 1 of which is shown) gave similar results to those previously described. After 24 hours, the cells recovered rapidly. Other mice were then injected with varying doses of antibody on the 6th day. The fall in cell count appeared to be directly related to the serum dose injected. Because the result was obtained with only a single observation at each point, the experiment was repeated twice more with similar results. This simple relationship does not exist with all tumors, and other dosage experiments showing enhancement at low serum doses will be presented in another section.

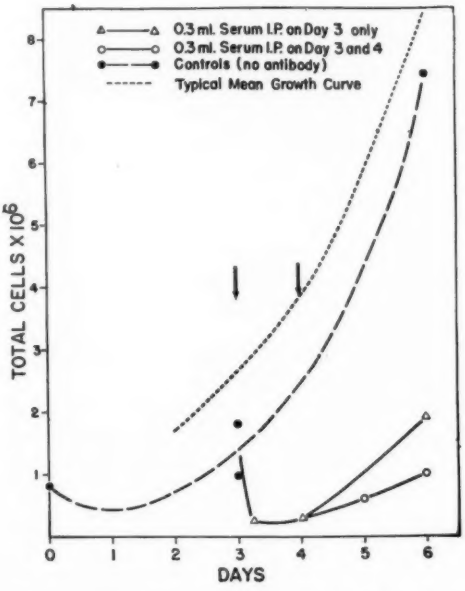
Maintenance of Growth Inhibition by Repetitive Injections of Antibody

As single injections of antibody would reduce the cell population but not eliminate all tumor cells, and as the survivors repeatedly showed signs of recovering from the effects of serum rather rapidly, experiments were set up in which antibody was injected into the same animals on successive days. In one series of experiments, antibody was given for 2, 3, or 4 successive days. Samples were taken 24 hours after each injection, and then at intervals, to follow recovery after treatment was stopped. The results are shown in text-figures 5 and 6. The values for the controls in the 2 experiments represented in text-figure 6 were close enough to fit a single line; in both experiments antibody was injected on the 2d and on subsequent days. In contrast to the results obtained with a single injection of serum, repeated doses restrained growth for as long as the injections were maintained. In the experiments illustrated, in which injections were given up to the 5th day, the count had begun to rise by the 7th day. When 4 injections were given, however, the count did not begin to rise until the 8th day, and was climbing sharply by the 9th day.

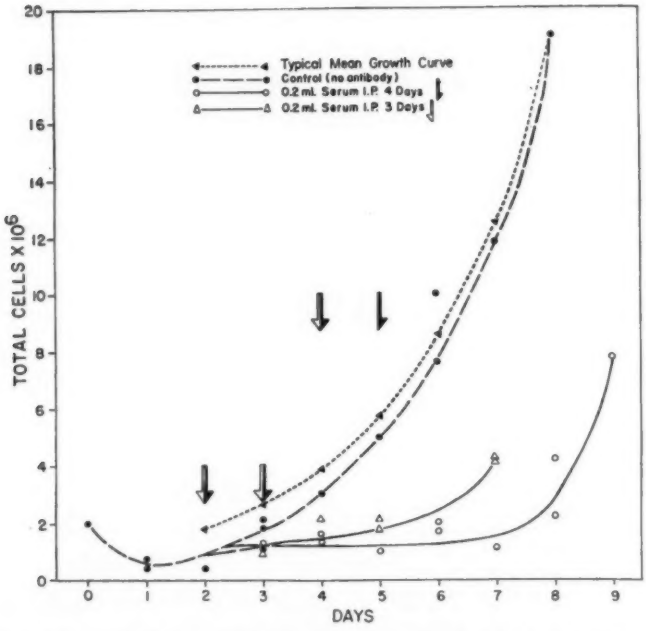
Mitotic counts were made of both the control and the experimental groups during these experiments. The mitosis rate in the control chambers stayed reasonably constant at about 2 percent, whereas in the experimental groups, there was close correlation between mitosis rate and the slope of the growth curve. The mitotic index was less than 0.25 percent during the phase of inhibition but approached the control values in the recovery stages.



TEXT-FIGURE 4.—Total populations of chambers containing the DBA/2 lymphoma after varying intervals of growth showing the regression line.



TEXT-FIGURE 5.—Prolongation of the effect of antibody by repeating the injection. DBA/2 lymphoma in DBA/1 mice; antibody, DBA/1-anti-DBA/2.

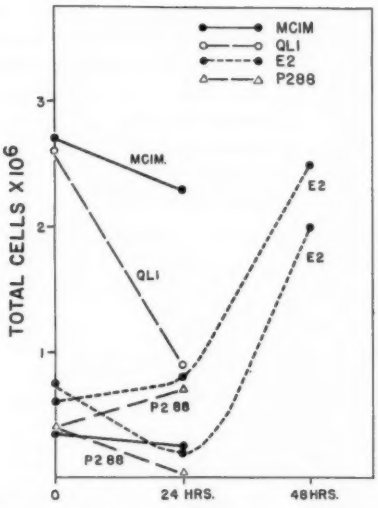


TEXT-FIGURE 6.—The inhibitory effect of 3 or 4 repeated injections of antibody on the DBA/2 lymphoma.

Because the antibody treatment in this group of experiments might have altered the cell population remaining in the chamber, samples taken after 3 to 4 days' treatment with antibody were transferred to fresh chambers in nonimmune DBA/1 mice and the antibody treatment repeated. Other cells were passed through weakly immunized DBA/1 mice for up to 9 transfers in an attempt to concentrate any resistant cells, and their response to antibody was tested when they were grown in chambers. In neither case was there evidence of any marked degree of antibody resistance; the majority of cells were still lysed by antibody and showed considerable sensitivity in a cytotoxicity test *in vitro*.

The Action of Specific Isoantibodies on Several Ascites Tumors

Short-term experiments have been run on a number of other tumors; the results are indicated in text-figure 7. In each experiment, the antibody had been prepared specifically against the tumor being tested. The DBA/1 Lymphoma QL1 (ascites) appeared to be as sensitive as the DBA/2 lymphoma, while inhibition rather than lysis appeared to be a feature of most of the other tumors. It is possible that 2 opposing activities are at work—lysis and increased rate of division. We have no general information on this, but it is not uncommon with some tumors such as Lymphoma P288 or E.L.4, at certain antibody levels, to find some ac-



TEXT-FIGURE 7.—Lytic and inhibitory effect of various isoantibodies against their specific tumor. All tumors were in the early stages of the rapid growth phase prior to injection. The experiments on E2 were performed in 129Rr/Ha mice with 129-anti-E2 antibody; on MC1M, in C57BL/Ha mice with C57-anti-MC1M; on P288, in DBA/1/Mirand mice with DBA/1-anti-P288; on QL1, in C57/Ha mice with C57-anti-QL1.

celeration or little obvious effect after 24 hours, but definite inhibition after 48 hours.

The inhibition obtained with Ehrlich carcinoma clone E2 and Fibrosarcoma MC1M is interesting because of the results of cytotoxicity tests *in vitro*. Although MC1M takes up trypan blue quite readily after treatment with immune serum and guinea-pig complement, these tumor cells do not readily lyse, and with E2 there is usually little staining or lysis but strong agglutination of the cells.

Experiments on C57BL Lymphoma E.L.4

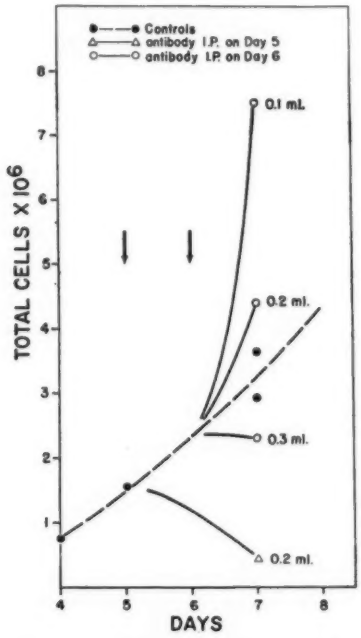
In the DBA/2 lymphoma, and in several other tumors tested, there is either a sudden fall in cell count, or a sharp flattening off in the growth curve. Two tumors have been exceptional; only one, E.L.4, has been studied in any detail.

The normal growth pattern of E.L.4 in diffusion chambers contrasts greatly with the rapid growth and cell density of the tumor grown as a free ascites. In the diffusion chamber, it grows slowly and produces fragmented and pyknotic nuclei even at low cell concentrations. The slope of its growth curve flattens off at a low level so that total concentrations of over 7 million in the chamber are exceptional. With the DBA/2 lymphoma, healthy populations of over 20 million can readily be obtained.

The extreme sensitivity of E.L.4 to antibody, shown repeatedly in passive immunity experiments (*3*), is less marked in the chamber. With

some relatively weak serums produced in DBA/2 or C3H mice, little effect was obtained until 48 hours, although with stronger samples the response was similar to that found in the other lymphomas.

In a dosage experiment (text-fig. 8) single injections of 0.2 ml. of the antibody tested caused a depression after 48 hours, as was expected. When a variable dose was given and the chambers sampled after 24 hours, there was a sharp increase in cell concentration with 0.1 ml., and, in contrast to the control population, the cells were healthy with few fragmented or dead cells. There was a slight increase with an injection of 0.2 ml. of the antibody and a moderate decrease with 0.3 ml. The experiment was repeated using the same serum with similar results.

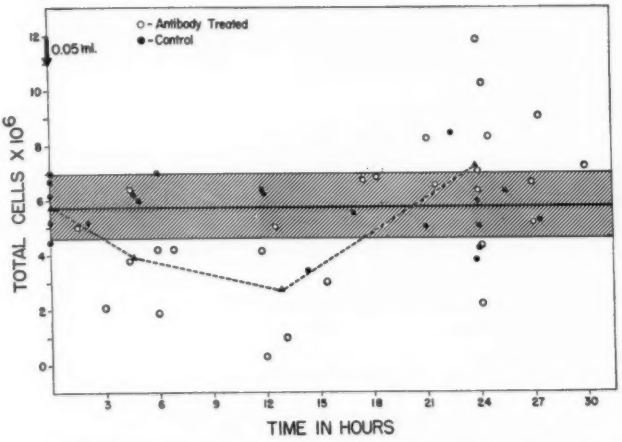


TEXT-FIGURE 8.—Inhibition, or growth acceleration of E.L.4 with varying doses of DBA/1-anti-E.L.4 isoantibody.

The enhanced rate of growth was not prolonged much beyond 24 hours, and in some experiments it appeared that the cell population was returning to normal within this time. Principal interest therefore centered around the early stages of enhancement, and the time interval between 1 and 30 hours after injection was more intensively studied. The potency against E.L.4 seems to vary considerably from serum to serum, and the balance between accelerated growth and inhibition appears to be precarious. One sample of DBA/1-anti-E.L.4 serum was used for the next 4 experiments, 2 in C3H/He, and 2 in DBA/1/Mi mice, with essentially similar results. It was established that, with this particular antibody,

inhibition followed the injection of 0.1 ml. and that the effect was marked within 4 hours, whereas 0.025 ml. had little effect. The results of injecting 0.05 ml. of antibody are shown in text-figure 9.

The growth curve of E.L.4 has changed somewhat since the first dosage experiments were performed, possibly because of a minor variation in technique. The tumor tends to grow more rapidly, but the total cell count is unchanged. Equilibrium is reached after about 4 days; the total cell count remains more or less constant, but there is a slight increase in the frequency with which the cells stain with trypan blue. The mitotic frequency has remained about the same for the duration of the experiment, with a mean of 1.64 percent [standard deviation (S.D.) 0.56].



TEXT-FIGURE 9.—Inhibition followed by moderate acceleration of growth of E.L.4 after treatment with small doses of isoantibody. *Hatched* area indicates standard deviation and the *heavy* line the mean of the controls. The mean of 3 experimental groups, 0 to 8 hours, 8 to 16 hours, 16 to 30 hours, respectively, is shown on the interrupted line joining them.

(Between 1,000 and 2,000 cells in an aceto-orcein squash preparation were counted for each determination.)

Considerably more variation in the cell counts was obtained in the experimental group. There was a modest fall starting soon after the serum injection, which was maintained for some 12 to 15 hours. After this the cell count rose steeply and in 4 chambers reached values higher than any in the controls. The general trend has been indicated by plotting the mean of values obtained soon after injection, 8 to 16 hours after injection, and all values after 16 hours. The trend was marked in each experiment but is to some extent obscured by a few chambers in which no acceleration occurred. This is not surprising as it is known that the balance between inhibition and acceleration is delicate. Experiments with this serum were not extended beyond 30 hours but, by analogy with other weak serums against E.L.4, a second fall might be expected.

Estimates of the number of viable cells and mitotic counts were made on each sample. There was considerable scatter in both sets of values, but the mean percentage of cells staining with trypan blue was twice that of the controls for the first 15 hours (13.9 as compared to 6.4%), but identical for the second 15 hours.

The over-all mitotic index for the controls was 1.64 percent, S.D. 0.56; and for the experimental groups, 1.85 percent, S.D. 0.64; (first 15 hours 1.93, S.D. 0.71; second 15 hours 1.80, S.D. 0.60). These slight differences are not significant, but there was certainly no diminution in mitotic activity, with antibody treatment, under the conditions of this experiment. The increase in cell number could be accounted for by a very slight increase in mitotic rate, a shortening of intermitotic time, and a slower rate of destruction. The cells in the antibody-treated animals were, in general, much cleaner than those in the controls, with fewer fragmented or pyknotic nuclei and fewer cells in which the cytoplasms had separated from the nucleus.

Discussion

The experiments reported show clearly that isoantibody is cytotoxic *in vivo* in the modified system used, and that mouse complement, although known to be ineffective in studies *in vitro*, appears adequate for the lytic reaction to proceed *in vivo*.

In confirmation of other reports (*2*, *4*), we find that only exceptionally does any cytotoxic effect occur *in vitro* without the addition of serum from a foreign species. A typical cytotoxicity test with fresh ascites cells from the DBA/2 thymoma would indicate that about 1 to 6 percent of cells stain after incubation for one-half hour in Ringer's solution; about 1 to 2 percent with serum from a guinea pig selected for low natural cytotoxicity; usually marked agglutination but little staining (from 2 to perhaps 10%) with isoantibody; but from 80 to 100 percent with isoantibody plus guinea-pig serum.

The trypan blue used as an indicator has a profound effect on the treated cells. Cells killed by physical means, or from an overcrowded population in an old tumor, stain intensely. The nucleus is sharply differentiated and the stained cell does not alter its morphology over considerable periods; cytoplasmic granules, presumably including mitochondria, are easily seen. However, cells treated with antibody and complement but without trypan blue tend to lose some of the clarity of the cell membrane and become somewhat ragged and less well defined. They appear to stay unchanged for at least 1 hour, probably for considerably longer. With the addition of trypan blue, an almost immediate and dramatic change occurs. The nucleus loses all definition and merges with the rest of the cell contents. All cytoplasmic detail is lost, the cell flattens out as an amorphous blob on the surface of the slide, and apparently all identifiable fragments soon become completely dispersed or dissolved.

These changes have been seen only in the presence of a strongly reacting

antibody and adequate complement—exceptionally, other changes are seen. The cells may balloon into large hollow vesicles in which the nucleus can sometimes be seen flattened against the wall before the cell ruptures and proceeds to lyse, or the blistering, characteristic of heterologous systems, sometimes occurs (*5*). Many cells, especially when mixed with a weaker antiserum, do not proceed to complete lysis but remain as well-differentiated, blue-staining entities; occasional cells in all but exceptional instances do not stain and remain apparently healthy and rounded with sharply defined cell membranes. The affected cells do not usually agglutinate, although clots are sometimes seen, and they do not adhere to the slide as normal cells do.

Trypan blue alone has little effect on normal cells. Any damaged cells stain almost immediately, and there is little increase in staining frequency for at least a half hour. The lytic effect of trypan blue is not simply due to a dilution of the protein solution in which the cells are suspended, though high concentrations of protein can interfere with uptake of the stain. Dilution with mammalian Ringer's solution has no effect apart from the normal deleterious effect usually found when cells are suspended in low protein-low nutrient solutions.

Direct observations on the lytic process seem to indicate that physical agents or low concentrations of antibody with complement simply increase the permeability of the cell membrane, thus allowing the dye to enter and stain either the nucleus or the nuclear membrane. Higher concentrations of antibody must alter the membranes of intracytoplasmic bodies. The effect of trypan blue on these membranes seems to be more complicated and may result in the liberation of high concentrations of enzymes which almost immediately autolyse the cell. Ellem (*6*) has recently shown that heteroantiserum in the presence of complement will greatly increase the permeability of the Ehrlich ascites cell to organic phosphates, and that marked swelling of the cells, probably due to osmotic changes, precedes lysis.

We do not know why all the potentially susceptible cells in a chamber are not killed by antibody. The two most obvious explanations of inadequate antibody levels or the development of antibody resistance by cell populations are easily excluded. The antibody concentration reached is almost certainly adequate since serum from the passively immunized homologous host or fluid from the chamber in which the cells were actually proliferating may have a hemagglutinin titer of over 1/1000 and is intensely cytotoxic when tested with fresh guinea-pig serum against normal ascites tumor cells. The remaining cell population does not appear to be antibody resistant since it will not grow when injected into strongly immunized mice and is also sensitive in a cytotoxicity test with known potent serum and fresh complement.

A third possibility, which seems to provide a partial explanation for many of our results, is that there is not enough complement for lysis of all the cells. Experiments designed to expand our knowledge of the availability of complement in a tumor-cell–antibody interaction are in

progress, and it appears that complement depletion is a major factor in limiting the extent of cell lysis, at least inside a chamber. Addition of normal mouse serum to the antibody at the time of injection increases the effect considerably, whereas mouse serum alone has no effect.

The finding that a proportion of the cells escapes damage by antibody conforms with earlier results on passive immunity to subcutaneous transplants in susceptible hosts. In the susceptible host, protection is readily demonstrated; but the tumor almost invariably appears after an interval that may be a few days or, in the case of E.L.4 which is unusually sensitive, after 10 days or occasionally even longer (*3*).

A certain minimum number of cells must be injected into a mouse of a foreign genotype before a local lesion develops, even in the absence of any pretreatment. The size of the lesion and the time it persists before regression depends on the dose. If the recipient has previously been treated with sufficient antibody, no lesion appears, although occasionally some animals develop a tumor several weeks after inoculation.

It has been thought that tumor cells transplanted to a foreign host might be at a metabolic disadvantage, and that a large inoculum was required because many of the cells die. The latter may be true, but there is no evidence from the diffusion-chamber studies that transplanted cells grow any better in the native strain, as the growth curves are almost identical. There is little gross infiltration of the graft area with host cells immediately after subcutaneous inoculation of a lymphoma, though a polymorphonuclear response may follow after about 48 hours; but even if a brisk response did occur, the method of disposal of the tumor cells would be interesting, as it has been stated that normal cells have no destructive activity toward a target (*7*).

Whatever the natural defense mechanism involved, it appears that a critical minimum inoculum is needed in a foreign genotype to produce a lesion. From the chamber studies it is obvious that antibody, even hampered by having to pass through the walls of a chamber, is capable of lysing many of the target cells. This activity in passively immunized foreign hosts would pull a subcutaneous inoculum below the threshold and no tumor would develop—the unknown natural defense eliminating the survivors. The immune response would in any event be slight and, in the presence of antibody cover, might be undetectable, as was in fact the case. In the compatible animal, a similar fall would ultimately lead to the appearance of a tumor though the onset would be delayed.

The presence of mitotic figures in normal proportions in most of the treated groups indicates that antibody usually has little effect on cell division. It is tempting to postulate that the reduction in activity in the animals treated with repeated injections of antibody is due to another process and that the X-type antibodies, which Gorer has stated to be noncytotoxic (*4*), act by inhibiting mitosis. Complement might not be needed for this activity, and this might be the primary effect in susceptible mice in which the *H-2* antibodies are quickly absorbed by normal tissues.

The accelerated growth, seen with low doses of antibody in some E.L.4

and P288 studies following the initial depression, appears to be associated with the generally improved condition of the cells after treatment. For some reason E.L.4 never grows well in the chambers. Even with cell concentrations of less than 1 million in the 0.5-ml. to 0.6-ml. chamber, many of the cells appear abnormal. The change within a few hours of antibody treatment is very obvious, and the proportion of morphologically damaged cells falls well below the proportion of such cells in control chambers. Many of the damaged cells must have been completely and selectively lysed. The well-being of the remainder could be due to release of metabolically needed substances from the lysed cells or even to the utilization of small amounts of antibody protein by the survivors. Certainly by the end of 24 hours the cells in the experimental chamber again appear to be little healthier than the controls. The diffusion chamber might be considered as a tool for the investigation of possible differences between the enhancing and cytotoxic effect of immune serums.

Studies on the action of antibody on susceptible cells were performed in chambers because the homograft reaction as a whole is too complex for systematic study. Good and his colleagues attempted to simplify the reaction by using patients with agammaglobulinemia, on which the antibody response is minimal (*8*), and others with Hodgkin's disease, in which a state of anergy exists (*9*). In both groups there is prolonged survival of the graft, and their latest conclusion is that an interplay of both cellular and humoral responses is essential. A similar inference could be drawn from our chamber studies, and our conclusion to date would be that, while antibody is undoubtedly effective in destroying a considerable proportion of the susceptible cells, nondiffusible factors are also involved. This may not be a specific effect and could be due to liberation, by polymorphonuclear cells and histiocytes, of enzymes ineffective against the intact cell but devastating to a cell membrane already injured by antibody and complement. However, there remains the possibility that antibody fails to destroy the target-cell population because of insufficient complement or inadequate amounts of part of the complement complex. Our current experiments are designed to clarify this point.

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Fetal Exposure to Diagnostic X Rays, and Leukemia and Other Malignant Diseases in Childhood[1]

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Summary

A survey of the comparative incidence of exposure to antenatal irradiation *in utero* and of the occurrence of leukemia and other malignant diseases in later childhood is reported. The case material consisted of deaths, before the age of 10 years, from leukemia and other malignant diseases, in Louisiana during the 5-year period 1951–55. The control cases were deaths from other causes matched for age, sex, race, and place of death. Information concerning the incidence of irradiation (diagnostic X rays during the last trimester of pregnancy) was obtained from professional sources, either from the doctor who delivered the mother or from the institution in which the child was born. The incidence of X-ray exposure was found to be as follows: leukemia cases, 26.9 percent; malignant disease cases, 28.4 percent; and control cases, 18.3 percent. These findings are in substantial agreement with those of Stewart *et al.*, in England. The findings lend emphasis to the need for minimizing exposure of the fetus to X rays, by careful determination of the indications for antenatal radiography and by attention to the technical procedures.—**J. Nat. Cancer Inst. 22: 1093–1104, 1959.**

In September, 1956, Stewart, Hewitt, Webb, and Giles (*1*) reported their preliminary findings from an investigation of the case histories of children who died of leukemia or other malignant disease, and of their mothers, before and during the relevant pregnancy. The material consisted of some 1,500 children who had died before the age of 10 years during 1953–55, in England and Wales, and paired control children. At that time information concerning about one third of the case material

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[4] We wish to express our thanks and to acknowledge the cooperation of the Officers of the Louisiana State and City of New Orleans Health Departments; the many physicians and superintendents of hospitals throughout the State who have contributed to this study; and Dr. L. E. Elveback for her advice and help in the statistical analyses.

had been collected, and preliminary analysis yielded results which the authors considered should be reported without delay: Although alike in other respects, one important difference existed between the case-history children and their controls, namely, that the number of mothers who stated they had had an X ray of the abdomen during the relevant pregnancy was almost twice as great for the cases as it was for the controls. By November 1, 1956, information from over 1,000 of the case-control pairs had been collected, and Stewart (*2*) still found that a history of antenatal abdominal X ray was obtained nearly twice as often from the mothers of children who had died from leukemia or cancer as from the mothers of live controls. Although the records had not been examined in detail, it appeared that the difference between the cases and controls with respect to the excess of "irradiated foetuses" was not due to difference in birth rank, social class, or maternal age. Stewart considered the most likely explanation to be that the total-body exposures to small doses of ionizing radiation before birth caused a significant increase in the risk of developing leukemia or cancer in childhood.

Stewart and her colleagues (*3*) published their final report in July, 1958. The findings concerning exposure to radiation, in that report, were that 13.7 percent of the mothers of children who died from leukemia or other malignant disease gave a history of abdominal irradiation during the relevant pregnancy, whereas 7.2 percent of the mothers of living, healthy control children gave this history.

That these findings and the tentative explanation have encountered widespread interest is reflected by numerous letters and articles published in the medical and lay press and addressed to a public which has recently become aware of the potential hazards of radiation. One objection to acceptance of Stewart's hypothesis is that association and cause are not synonymous. Although a statistical association of irradiation with the later development of leukemia may be demonstrated, it cannot for that reason alone be considered a causal association. If, however, a causal relationship does exist it might be expected that the association would still be demonstrable in different environmental circumstances. Hence the present study was undertaken, shortly after the preliminary report by Stewart *et al.* (*1*) was published, to determine whether the association between antenatal exposure to irradiation and the development of malignant diseases in children in England was demonstrable in the State of Louisiana.

Procedure

The case histories of all children under 10 years of age certified to have died, in Louisiana, of leukemia and other malignant diseases during 1951–55 and of a group of 608 "control" children in Louisiana who had died from other causes during the same period were reviewed with the cooperation of the Health Departments of the State of Louisiana and of the city of New Orleans. It was predicated in this study that the relevant information be obtained from professional sources; no direct inquiry was

to be made of the relatives without the approval of the doctor concerned because of the privileged nature of such information in the State of Louisiana.

The death certificates were inspected and the following information obtained: the place of death, the usual residence at time of death, the mother's maiden name, the doctor's name, whether or not there had been an autopsy, and the place and date of birth. The corresponding birth certificates were then sought and from these the following data were obtained: confirmation of the mother's maiden name, residence at the time of birth, the duration of pregnancy, presence of malformation, the weight at the time of delivery, the attendant at the delivery, and the institution in which the child was born. All "out-of-State" birth certificates were obtained from the appropriate health departments. A total of 254 of the birth certificates could not be located or were useless because of:

1) Foreign birth	4
2) Midwife delivery	95
3) Adoption and change of name	85
4) Illegibility of the attendant's signature	70

An inquiry addressed to the doctor who delivered the mother requested information regarding exposure to abdominal or pelvic X irradiation during the gestation of the child, and the purpose, time, and number of exposures; information regarding neonatal irradiation was also requested.

No replies were received to 97 inquiries (16%). This was in a number of instances owing to the removal or the death of the doctors who had delivered the mothers; the cooperation of the doctors, on the whole, was very gratifying.

The numbers were further diminished for the following reasons: 1) The diagnosis in 6 cases of leukemia and 2 of malignant disease was proved to be erroneous; all these children were under 2 years of age, and 4 were premature infants. 2) Because these 8 cases were removed from the leukemia and other malignant disease categories, the control category was preponderant for the ages 0 to 1 year and 1 to 2 years, though less so for the latter. It was therefore decided that the control group be decreased by 35 children under 2 years of age.[5]

Table 1 shows the initial groups of cases, the effect on them of loss of cases for the reasons mentioned, and the final groups remaining for further analysis.

The next desideratum was that the remaining leukemia, other malignant disease, and control categories should match one another with

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[5] The following were eliminated:

Deaths associated with prematurity	5
Deaths due to erythroblastosis	2
Deaths associated with congenital anomalies (hydrocephaly, meningocele, etc.) up to the age of 2 years	28
Total	35

It became apparent later that 7 of the 35 children (20%) had been exposed to irradiation *in utero;* the final calculation of the incidence of exposure in the control group was not materially affected by the elimination of these cases.

TABLE 1.—*Cases eliminated and cases remaining (by race and sex), after scrutiny of birth certificates and replies to inquiries*

Group	Race and sex	Initial group	Birth certificate not located	Midwife delivery	Cases eliminated	No reply	Final group
Leukemia.....	WM	56	2	3	2	8	41
	WF	39	1	0	2	7	29
	CM	13	4	4	1	1	3
	CF	9	2	1	1	0	5
Subtotal.....	.....	*117*	*9*	*8*	*6**	*16*	*78*
Other malignant diseases	WM	49	9	1	1	4	34
	WF	43	7	1	1	7	27
	CM	18	6	2	0	2	8
	CF	17	8	3	0	1	5
Subtotal.....	.....	*127*	*30*	*7*	*2**	*14*	*74*
Controls	WM	258	42	26	14	34	142
	WF	189	28	22	11	23	105
	CM	87	26	17	2	2	40
	CF	74	24	15	8	8	19
Subtotal.....	.....	*608*	*120*	*80*	*35†*	*67*	*306*
Total.....	.....	852	159	95	43	97	458

*Errors in diagnosis.

†Adjustment of control series because of imbalance in age distribution caused by elimination of 8 cases of mistaken diagnosis.

respect to characteristics other than that under investigation. The categories were therefore compared with one another as follows:

Age, sex, and race distribution: There was a greater number of cases for the ages 2 to 5 years in all 3 categories and the distributions above and below this peak matched fairly well (table 2). This, as explained, was due, in part, to the elimination of 35 control cases under the age of 2 years. In the leukemia category, white male children comprised 52.6 percent and white females 37.2 percent; in the other malignant disease category, white males comprised 45.9 percent and white females 36.5 percent; of the 306 control children used, 46.4 percent were white males and 34.3 percent were white females.

Place of death: The control cases had been originally chosen on a matching basis for the place of death, *i.e.*, the parish in which death occurred. Those remaining for analysis were still well matched (table 3); 68 percent of the patients with leukemia, 62 percent of the patients with malignant disease, and 61 percent of the control patients had died in the hospital. More of the control patients, however, had died in a rural area than the patients in the other 2 categories. This was partly due to the greater number of accidental rural deaths. There were 53 fatal rural accidents (43% of the rural deaths) and 57 fatal urban accidents (31% of the urban deaths).

TABLE 2.—*Age, sex, and race distribution of leukemia, other malignant diseases, and control cases exposed and nonexposed to antenatal irradiation*

Group		Race and sex	Age in years: 0–1	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	Total
Leukemia (78)	Exposed (21)	WM	1	2	2	2	1	1		2		1	12
		WF			1	1	2	1		3			8
		CM											
		CF	1										1
	Nonexposed (57)	WM	2	2	3	6	5	3	1	4	3		29
		WF	2		3	4	2	2	5	2	1		21
		CM	1				1		1				3
		CF		2	1		1						4
Other malignant diseases (74)	Exposed (21)	WM	4			4	1	2			1	1	13
		WF	1	1				3	1	1			7
		CM							1				1
		CF											
	Nonexposed (53)	WM	2	1	1	6	5	1	2	1		2	21
		WF	1	5		2	2	4	2	4			20
		CM			2	1	4						7
		CF		2	1	1		1					5
Controls (306)	Exposed (56)	WM	4	1	3	5	4		2	5	2		26
		WF	6	1	2	4	1	3	2	1		2	22
		CM			1	1				1		1	4
		CF	4										4
	Nonexposed (250)	WM	9	8	18	23	19	12	8	12	5	2	116
		WF	5	5	5	13	12	14	10	12	3	4	83
		CM	5	1	3	6	10	3	6	1		1	36
		CF	2	5		2	1	3		1		1	15

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TABLE 3.—*Distribution with respect to place of death: hospital, rural, or urban*

Group	Total	Deaths in hospital (*percent*)	Rural deaths (*percent*)	Urban deaths (*percent*)
Leukemia	78	67. 9	26. 9	73. 1
Other malignant diseases	74	62. 2	25. 6	74. 3
Controls	306	60. 8	40. 5	59. 5

Place of birth: The place of birth had not been considered in selecting the control cases. Table 4 shows that the rural-urban distribution for the leukemia and control categories is in close agreement.

TABLE 4.—*Distribution with respect to place of birth: hospital, rural, or urban*

Group	Total	Births in hospital (*percent*)	Rural births (*percent*)	Urban births (*percent*)
Leukemia	78	100	38. 5	61. 5
Other malignant diseases	74	97. 3	48. 6	51. 4
Controls	306	95. 4	39. 2	60. 8

Results

The replies to the questionnaires were analyzed to determine the incidence of exposure to diagnostic X rays *in utero*, with the results shown in table 5. The diagnostic X rays comprised abdominal or pelvic X rays taken for obstetric reasons during the last trimester of pregnancy, in all except 2 control patients who had an intravenous pyelography during the 2d month of their pregnancies.

Among the children who had died from leukemia and from other malignant diseases, the incidence of exposure to this type of irradiation during fetal life was found to be 26.9 percent and 28.4 percent, respectively, whereas the incidence among control children who had died from other causes was 18.3 percent. These differences, while suggestive, do not justify a conclusion that the relative frequencies of exposure to antenatal irradiation differ in the populations represented by the sample data. It must be recognized, however, that with the sample sizes of this study the detecting power against differences of the magnitude found by Stewart *et al.* (*3*) is low. It is the similarity of the results in the two studies which is of primary interest.

Table 6 shows the incidence of exposure for children in the age groups 0 to 4 years old and 5 to 9 years old at the time of death. In the control cases the incidence in the 5- to 9-year-old group is lower than in the 0- to 4-year-old group. There is no such difference in the incidence of exposure between the two age groups in the leukemia category and a

TABLE 5.—*Incidence of exposure to diagnostic irradition* in utero

Deaths from:	White				Negro				All	
	Males		Females		Males		Females			
	Number exposed / Total No.	Percent exposed	Number exposed / Total No.	Percent exposed	Number exposed / Total No.	Percent exposed	Number exposed / Total No.	Percent exposed	Number exposed / Total No.	Percent exposed
Leukemia...	12/41	29	8/29	28	0/3	0	1/5	20	21/78	26.9
Other malignant diseases.....	13/34	38	7/27	26	1/8	13	0/5	0	21/74	28.4
Other causes —controls.	26/142	18	22/105	21	4/40	10	4/19	21	56/306	18.3

contrary difference in the other malignant disease category. Stewart *et al.* (*3*) interpreted similar findings to imply that the consequences of exposure to irradiation during fetal life were not exhausted by the 10th year.

TABLE 6.—*Incidence of exposure to diagnostic irradiation* in utero *of children who died at ages 0 to 4 years and 5 to 9 years*

Group	Deaths 0 to 4 years		Deaths 5 to 9 years	
	Number	Percent exposed	Number	Percent exposed
Leukemia	48	27	30	27
Other malignant diseases	47	23	27	37
Controls	189	20	117	16

The number of roentgenograms reported to have been taken are compiled in table 7. This represents a very crude estimate of the dose of X rays. While it is not possible to make statistical inferences from this table, it is included because of its remarkable similarity to table 8 in the paper of Stewart *et al.* (*3*).

In the leukemia series one child included in the nonexposed group was a twin who, at birth, had an enlargement of the thymus gland. One of the twins received X radiation to the thymus. They were, at the time, designated Baby 1 and Baby 2, and it has proved impossible now, 12 years later, to establish which twin was irradiated.

The cytological diagnoses of the leukemia cases and the sites of the other neoplasms were as follows:

	Exposed	Nonexposed
Leukemia	21	57
Lymphogenous	13	32
Myelogenous	1	4
Monocytic		2
Stem-cell, aleukemic and undifferentiated	7	19
Other malignant diseases	21	53
C.N.S.	6	10
Kidney	4	10
Neuroblastoma	2	9
Lymph nodes	4	10
All other sites	5	14

Birth rank.—Data on birth rank are incomplete as birth rank was not recorded on all the birth certificates located. For the total number of cases in each category in which this information is available, the following are the percentages of first-born children:

Leukemia	39.1% of 87 children
Other malignant diseases	31.5% of 89 children
Controls	25.2% of 424 children

Maternal age.—The mean maternal ages in all cases for which parity is known are shown in table 8. When all mothers were considered, no significant differences existed between the ages of the mothers in the different categories. The mothers of first-born children who died of leukemia, however, were older than the mothers of first-born control children ($P = 0.016$); the mothers of first-born children who died of other malignant disease were not older than the mothers of first-born control children. None of the mothers of first-born children was over 40 years old. Mothers of first-born control children exposed to irradiation *in utero* were older than mothers of first-born nonexposed controls ($P = 0.034$). These findings might suggest that the significance of maternal age rested in the increased likelihood of X radiation during pregnancy in older primigravidae. The large series of Stewart *et al.* (*3*) did not support such a suggestion, and the numbers involved in the present study were too few to enable valid conclusions to be drawn. One child in the leukemia series was a mongoloid; the mother's age was 21 years.

TABLE 7.—*Distribution of roentgenograms among cases and controls exposed to irradiation* in utero *during the relevant pregnancy*

Number of roentgenograms	Cases		Controls		Ratio Percent cases
	Number	Percent	Number	Percent	Percent controls
0	110	72. 4	250	81. 7	0. 88
1	19	12. 5	30	9. 8	1. 27
2	10	6. 6	13	4. 3	1. 55
3	4	2. 6	7	2. 3	1. 15
4 or more	8	5. 2	5	1. 6	3. 25
(Unknown number)	(1)	(0. 7)	(1)	(0. 3)	(2. 33)
Total.....	152	(100)	306	(100)	1. 00

Discussion

In the study done by Stewart and her colleagues (*3*), the control cases were living, healtby children and the relevant information was obtained by interviewing the mothers. In the study reported in this paper, the control cases were children who had died from other causes, and the information was obtained from the doctors who delivered the mothers and by cross reference to hospital records, where available. Despite these differences in method, the findings with respect to the incidence of antenatal irradiation are of a strikingly parallel order. Stewart *et al.* found that the relative risk of death from malignant disease in the exposed group compared to that of the nonexposed group was 2.05.[6]

[6] Relative risk (R) $= \frac{p_e(1-p_c)}{(1-p_e)p_c}$, where p_e = percentage of disease group exposed and p_c = percentage of control group exposed, *e.g.*,

$$R = \frac{13.7 \times 92.8}{7.2 \times 86.3} = 2.05$$

data from Stewart *et al.* (*3*)].

In the present study the corresponding figure is

$$R = \frac{27.6 \times 81.7}{18.3 \times 72.4} = 1.7$$

(for the leukemia *versus* control deaths $R = 1.64$).

TABLE 8.—*Mean age of all mothers and mothers of first-born children in the 3 categories*

Group	All mothers				Mothers of first-born children		
	Number*	Mean age, $\bar{x}$	$SE_{\bar{x}}$	Percent over 40 years	Number*	Mean age, $\bar{x}$	$SE_{\bar{x}}$
Leukemia	87	26. 0	0. 608	1. 1	34	23. 8	0. 917
Other malignant diseases	89	25. 0	0. 622	2. 2	28	21. 0	0. 799
Controls	424	25. 6	0. 302	2. 8	107	21. 4	0. 436
Exposed controls	52	26. 3	0. 943	3. 8	23	22. 9	1. 009
Nonexposed controls	224	24. 9	0. 387	2. 2	55	20. 5	0. 517

*Total number of cases for whom information regarding parity is available.

Since the control group in this study is composed of deaths rather than living children, R must be interpreted as the relative risk of death that is due to malignant disease among deaths rather than, as usually, the relative risk of death that is due to malignant disease in the populations under study. However, if it may be assumed that the proportion of the exposed population under 10 years old can be estimated as 0.183, then the relative risk of 1.7 may be interpreted in the usual way as being the ratio of the risks in the exposed and nonexposed population. If the proportion exposed in the population is lower than 0.183 (derived from 18.3% observed among the control deaths) the relative risk would be increased. The use of antenatal X rays appears to be more common in Louisiana than in England. In Louisiana, however, the actual percentage of pregnant women who had X rays is probably lower than that suggested by the figures given in table 5. It is unlikely, perhaps, that the patients delivered by midwives had had antenatal X-ray examinations. If all these deliveries were included as "nonexposed" cases, the incidence of antenatal abdominal irradiation in the control cases would be 14.5 percent, and for the leukemia and other malignant disease cases, 24.4 and 25.9 percent, respectively. The relative risk would become 1.97.

These findings were not invalidated by Kjeldsberg's conclusions (*4*) that antenatal irradiation was not responsible for the occurrence of leukemia in 55 children in a particular clinic in Norway since many other factors may be involved in leukemogenesis. Stewart *et al.* found that the incidence of other prenatal events, such as virus infection, threatened abortion, and maternal age, were significantly higher for the case children who died from leukemia; the numbers were too few, however, to permit making valid inferences.

The low incidence of leukemia among Negro children is a puzzling feature: they accounted for 37.2 percent of the children under 10 years old in Louisiana in 1950 but for only 18.8 percent of the deaths from leukemia among children under 10 years of age during 1951–55. During the same period, 27.6 percent of the deaths from other malignant diseases among children under 10 years of age occurred in Negro children. It is difficult to believe that, with respect to leukemia, the relevant diagnoses are not made since charity hospital facilities are freely available to, and commonly made use of by, the Negro population. MacMahon and Koller (*5*) found the incidence of leukemia in Negroes of all age groups equal to that of non-Jewish whites in New York City. Lea and Abbatt (*6*) have discussed some of the difficulties encountered in making a true estimate of the incidence of leukemia in a population and concluded that the certified death rates were more likely to be minimal rates than overestimates. At the present time we have no useful explanation to offer for the apparently low incidence of leukemia in Negro children. It should be noted, however, that there exists among Negro children a higher competitive risk of death from other causes. Thus, in 1951, the Negro infant mortality rate in Louisiana was double that for white infants; the Negro infant mortality rate from infectious diseases was

treble that for white infants; and the death rate from accidents for Negro children who were 0 to 10 years old was double that for white children. If such competitive risks are greater for this age group, and the leukemia risk is the same for both Negro and white children, it appears that the competitive risks may contribute to the "deficiency" of leukemia deaths among Negro children.

Gunz, Borthwick, and Rolleston (7) have reported from New Zealand the case of a child who received excessive doses of diagnostic radiation before birth and died from leukemia when 10 months old. This case was of interest because the authors calculated the dose of radiation received by the fetus and showed that, by modifications in the technique of radiography, which have since been introduced, the dose received by that fetus might have been reduced by 92 percent. These and similar findings demonstrate the necessity for a careful and continuous scrutiny of the clinical indications in radiography during pregnancy and of the techniques employed so that antenatal exposure to X rays is kept to a minimum.

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Spray Technique for Preparation of Cytologic Specimens for Automatic Scanning Machines[1]

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Summary

A method of preparing vaginal-cervical material for automatic scanning by the Cytoanalyzer, or similar machines, is described. The method involves fixation of the aspirated cells in solution followed by sieving the specimen through a 53-μ sieve. After centrifugation and decantation of the supernatant, the cells are resuspended in 5 cc. of absolute alcohol, placed in an atomizer, and sprayed onto a selected glass slide. The slides are then stained by the Feulgen reaction without counterstain. This method of preparation allows the machine to evaluate each cell accurately, and has reduced the machine error of the Cytoanalyzer to such an extent that a correct analysis of necessary electronic improvements can now be made for the final development of a successful clinical scanning machine. The results of a controlled clinical trial of vaginal aspirations processed in this manner will be published in the near future.—**J. Nat. Cancer Inst. 22: 1105–1117, 1959.**

One of the greatest single obstacles to the advancement of automatic screening of vaginal-cervical material by scanning machines has been the lack of a satisfactory method of preparing the specimens. Most of the difficulties encountered are directly related to the fact that the specimens obtained by vaginal aspiration or cervical scraping contain generous quantities of mucus, clumps of epithelial cells held together by intercellular matrix, clumps of white blood cells held together by surface attraction, and epithelial cells in all the different stages of degeneration. The cells are of irregular sizes and shapes and frequently have extensive cytoplasm which folds upon itself causing areas of increased density other than those due to nuclei.

Even when the cells are not counterstained, the cytoplasm will absorb enough of most nuclear stains to cause considerable absorption of light, so

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[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

that multiple cytoplasmic overlaps appear as a nucleus to a scanning machine with optical density as a criterion.

The Cytoanalyzer, type 401, is an area and density measuring instrument, which represents perhaps the most ambitious effort to date to develop a machine that would satisfactorily scan specimens of vaginal-cervical material. Its development is an attempt to separate the definitely negative specimens from those which might have some abnormalities. The machine would be considered practical and economically feasible for large-scale screening if it could operate at a level of 50 percent, or less, false positives and a near zero level of false negatives.

In order to discuss the problems of smear preparation for the Cytoanalyzer, it is necessary to describe briefly its capabilities. The instrument subjects all events in a field to a process of logical selection and accepts or rejects those events for evaluation on the basis of the following rules: (*a*) The nucleus/cytoplasm contrast must exceed a specified value. (*b*) The cytoplasmic absorption must be less than a specified value. (*c*) Specific requirements for continuity of shape are imposed. (*d*) The area of the nucleus must be less than 254 μ^2, and the diameter of the nucleus must be less than 18 μ in the X direction. (*e*) The nucleus must be less than 20 μ in the Y direction. (*f*) Cells overlapping the edge of the field are ignored (*1*). Any particle on the slide which meets these selection rules is evaluated and counted into 1 of 5 zones depending upon the area of the nucleus and the percent of light absorbed by the nucleus. There is also a "reject" zone for those particles on the slide that do not meet the necessary criteria required to be counted as a cell. Such events as clusters of cells or patches of debris usually fall into the reject zone.

The boundaries of size and density of Zone 1 are such that most leukocytes will be counted into this zone. The boundaries of Zone 2 correspond to most normal cervical epithelial cells. Those cells that meet the size and density requirements of atypical and malignant cervical epithelial cells will be counted into Zones 3 and 5. Zone 4 is for those cells that are enlarged but have lightly stained chromatin. Postmenopausal women with atrophic changes of the cervix frequently have cells of this size and density (*see* text-fig. 1).

A fairly advanced stage in the electronic development of the machine was reached before it became evident that adequate preparation of the specimen for automatic scanning was a task which might cause considerable difficulty. This paper deals with the development of a new method of preparing vaginal-cervical material for automatic scanning by the Cytoanalyzer, or similar machines, along with a brief discussion of previous attempts that were found to be unsuccessful.

Background material.—Previous investigators had shown that the routine Papanicolaou method of preparing vaginal aspirations was unsatisfactory for automatic scanning by the Cytoanalyzer (*2*, *3*). Errors in classification resulted in an average of 7.2 percent of the epithelial cells being scored in the suspicious zones when actually no abnormal cells were present on the slides. In persons with early cervical malignancy,

TEXT-FIGURE 1.—Size and density parameters of the Cytoanalyzer 401.

atypical cells frequently account for only 0.5 percent of the total epithelial cells present, so that the error rate must be below this figure for the machine to differentiate between the positive and negative cases (*4*).

Causes of errors in evaluation of cells in the order of their importance were: (*a*) cytoplasmic overlaps, (*b*) overlapped epithelial cell nuclei, (*c*) darkly stained cytoplasm, (*d*) leukocytes overlapping epithelial cell nuclei, (*e*) clumps of leukocytes, (*f*) focus, and (*g*) artifacts and debris.

Analysis of the capabilities of the machine and causes of incorrect evaluation of cells led to the belief that the ideal slide preparation of vaginal-cervical material for automatic scanning by a machine such as the Cytoanalyzer would be as follows: (*a*) A generous number of cells on the slide in an even distribution with minimal overlapping of nuclei. (*b*) Minimal clumping of cells—either epithelial or white blood cells. (*c*) The cytoplasm would be unstained and therefore would absorb very little light. (*d*) Mucus would be removed. (*e*) Erythrocytes would not be stained. (*f*) Malignant cells present in the original aspiration would not have been lost in processing. (*g*) The slides could be accurately screened by technicians as well as by the machine.

Materials and Methods

Removal of Clumps of Cells From Vaginal Aspirations and Cervical Scrapings

Clumped cells are present in large numbers in vaginal aspirations and cervical scrapings and are held together by (*a*) surface attraction, (*b*) mucus, and (*c*) intercellular cement. Those held together by surface attraction alone are predominantly leukocytes and can be separated to some extent by suspending the cells in physiological saline solution and agitating the suspension. Mucous clumps of epithelial cells and leukocytes are broken up at the same time. The epithelial cells, however, are frequently held together by intercellular cement and are extremely difficult to separate. Trypsin, pepsin, hyaluronidase, streptokinase, and streptodornase were tried, without success, to break the bonds between the epithelial cells.

An attempt was made to remove the clumps by the use of a wire sieve of small-pore diameter. Though it has been considered advantageous for screening to have all the cells in the specimen left on the slide, there are several reasons why removal of the clumps by sieving might improve the specimen for automatic scanning by a machine that makes a final decision on the basis of percentages: *a*) Several investigators have shown that malignant cells have decreased mutual adhesiveness (*5*, *6*). If this is true, one might expect that more clumps of epithelial cells in the specimen would be normal rather than malignant, and by removing a relatively higher percentage of normal epithelial cells, sieving would actually enrich the ratio of malignant cells to normal cells to be put on the slide. *b*) Many of the malignant cells in the specimen are deeper parabasilar cells, which have a large nucleus but very little cytoplasm. The total

diameter of these cells is less than the total diameter of the normal superficial epithelial cells and are therefore less likely to get caught on top of the sieve. This also tends to enrich the ratio of malignant cells to normal cells. It is true, however, that these parabasilar cells frequently are seen in small clumps which might cause an opposite effect. *c*) The sieve removes a large amount of mucus in the specimen and a cleaner preparation results.

To evaluate the theoretical advantages listed above, vaginal aspirations and cervical scrapings were obtained from patients with known cervical malignancy. The cells were washed from the pipette into 50 cc. of physiological saline. Cervical scrapings obtained from the same patients were also suspended in 50 cc. of normal saline. The specimens were poured through U.S. Standard Sieves [5] whose screen-pore sizes were 53 μ in diameter. These specimens were then filtered onto Millipore filters [6] and stained with the routine Papanicolaou technique (*7*).

After sieving the specimens, each sieve was inverted and 50 cc. of physiological saline was poured back through the sieve to obtain the material that remained on top of the screen. This "wash-back" material was then filtered onto Millipore filters and stained as described. The number of normal, atypical, and malignant cells on each filter was counted, which enabled us to calculate the percentage of abnormal cells that went through the sieve and the percentage of cells that remained on top of the sieve. Results indicate that the percentage of abnormal cells in both vaginal aspirations and in one of the cervical scrapings was slightly increased by sieving, but the percentage was slightly decreased in the other cervical scraping. Most of the clumps of cells remained on top of the sieve, and most of the single cells were washed through it (table 1). Therefore, we conclude that the sieving of vaginal aspirations and cervical scrapings, through a 53-μ sieve, successfully removes most of the clumps of cells without materially changing the ratio of atypical and malignant cells to the normal cervical epithelial cells.

Obtaining a Monolayer of Cells on a Glass Slide

The specimen must be put onto the glass slide in such a way that the cells are sufficiently close to each other to enable the machine to make a rapid scan of a large number of cells, but not so close that there is a high probability of overlap. There should be a homogeneous spread of cells over the slide with a fairly constant number of cells per unit area.

The method considered to be most satisfactory for solving the distribution problem involved the use of an atomizer to spray the cells onto the slide.[7]

Specimens are obtained by vaginal aspiration with glass pipettes. Each pipette is rinsed with 5 cc. of physiological saline, and the suspension gently agitated. Forty-five ml. of absolute alcohol is then added to the

[5] U.S. Standard Sieve Series, No. 270. W. S. Tyler Company, Cleveland 14, Ohio.
[6] Millipore filter #SM. Millipore Filter Corp., Watertown, Mass.
[7] Suggested by George Z. Williams, M.D., Chief of Department of Clinical Pathology, National Institutes of Health, Bethesda, Md.

TABLE 1.—*Effect of 53-μ sieve on vaginal aspirations and cervical scrapings*

Case No.	Percent of cells that went through 53-μ sieve	Percent of abnormal cells that went through 53-μ sieve	Ratio of abnormal to normal cells in complete original specimen	Ratio of abnormal to normal cells that went through 53-μ sieve
Vaginal aspiration				
1. Class 111b	97. 5	98. 6	1/5. 06	1/5. 05
2. Class V	68. 0	71. 3	1/1. 21	1/1. 18
Cervical scrapings				
1. Class 111b	98. 6	94. 4	1/2. 92	1/2. 93
2. Class V	45. 0	59. 0	1/4. 73	1/3. 27

specimen, which gives a final concentration of 90 percent alcohol that is adequate for fixation. Agitation of the specimen in 5 cc. of physiological saline prior to fixation breaks up many of the clumps held together by mucus and surface attraction.

The suspension of fixed cells is poured through a 53-μ wire sieve and collected in a 50-ml. test tube, which is then placed in a centrifuge machine and spun at 300 $\times$ g for 10 minutes. The supernatant is decanted, and the small pellet resuspended in 5 cc. of absolute alcohol. (The purpose of this step is to reduce the amount of fluid to be sprayed onto the slide.) The 5-cc. specimen is then placed in an atomizer [8] and sprayed onto a glass slide from a distance of about 4 inches, with the smallest amount of air pressure necessary to give an even spray. This is usually about 10 to 12 mm. of mercury pressure. The glass slide may be placed at an approximate angle of 60° from the horizontal, to facilitate spraying. After spraying, the excess alcohol is allowed to drain and evaporate before the slide is placed into acetic-formalin fixative. The cells have already been fixed in 90 percent alcohol so that there is no harmful drying effect. It is interesting and somewhat surprising that, with the spray technique, the cells adhere without a previous coating of albumin on the slide (*see* figs. 1 and 2).

This technique gives excellent distribution of cells on the slide without apparent significant damage to the cells. It has been found, however, that with air pressures above 16 mm. of mercury many of the cells will show frayed cytoplasm and ruptured nuclei. At 10 to 12 mm. of mercury pressure, the average number of split epithelial cell nuclei is only 1 per thousand. The occasional split nuclei do not cause machine or technician's errors.

The Millipore-filter technique of preparing specimens for cytologic examination, as described by Seal (*8*), was also used in an attempt to solve the distribution problem, but did not fulfill all the necessary criteria for automatic scanning.

Advantages of the Millipore filter were (*a*) excellent distribution of cells, and (*b*) an adequate number of cells on the slide, even from the scantiest specimen. Disadvantages were (*a*) expense of filters, (*b*) difficulty of handling and mounting the fragile filters, (*c*) wrinkles in the filter mounted on the glass slide which caused improper focus, (*d*) debris and artifacts were accentuated by the filter, which retained even the smallest amount of trash present in the specimen. Disadvantages (*c*) and (*d*) caused a significant number of improper entries into the higher or suspicious zones of the machine.

Another method, tried in an effort to get the cells onto the slide in an even manner, consisted of resuspending the specimen in only 0.5 cc. of 95 percent alcohol after fixing, sieving, and centrifuging as described earlier. A pipette was used to drop the specimen of suspended cells onto a glass slide. The alcohol evaporated causing the cells to settle randomly onto the slide. The "drop" method gives very nice prepara-

[8] Calab atomizer, Size D. California Laboratory Equipment Company, Berkeley 7, Calif.

tions but results in a higher percentage of nuclear overlaps than does the "spray" technique. Therefore it is not considered useful for automatic scanning by the Cytoanalyzer. The "drop" method without sieving or centrifugation might prove to be as accurate for technician screening as the standard "swab" or "pull" methods, and probably would be easier to screen because of the improved distribution of cells (*see* fig. 3).

Staining of Cytologic Material for Automatic Scanning

As previously mentioned, for best results with the Cytoanalyzer, it would be desirable to stain the nuclei moderately dark and to stain the cytoplasm at a minimum. In terms of percent absorption of light, the nuclei of the epithelial cells should absorb approximately 50 percent of the light and the cytoplasm less than 15 percent. With this N/C absorption ratio it would take 3 to 4 superimposed cytoplasmic overlaps to cause a machine error. That amount of overlap is unusual with the sieve and spray technique.

The stain that most nearly meets the requirements above is the Feulgen. The Schiff reagent is prepared by the traditional method (*9*), and the staining procedure is as follows: (*a*) Take slides from fixative and place in water (preheated) at 60° C. for 1 minute. (*b*) Place in normal hydrochloric acid (preheated) at 60° C. for 10 minutes. (*c*) Immerse in Schiff reagent for 30 minutes at room temperature. (*d*) Wash 1, 10, and 1 minutes in 3 successive baths of water, acid-alcohol, and absolute alcohol. (*e*) Clear in xylol for 1 minute and mount in permount or other mounting media. The epithelial cell nuclei are stained a deep pink by this technique and have a light absorption of 30 to 70 percent. The cytoplasm is usually invisible to the eye and causes 0 to 10 percent light absorption as measured by the machine. The intensity of the stain is constant enough to obviate the necessity of restandardizing the machine for each slide.

Without a counterstain, some technicians experience difficulty in screening the slides, stained by the Feulgen method, for the Cytoanalyzer. Most cytology technicians have been trained in the use of Papanicolaou stains and they find the less intense Feulgen a little harder to evaluate. It is thought, however, that as technicians become accustomed to the stain, proficiency will increase. It is also possible to equip the machine with filters to block out the spectrum of a counterstain, such as dinitroflourobenzene, which has no spectral overlap with the Feulgen. The blocking filters would permit counterstaining of the cells for the benefit of the technicians without causing machine errors due to cytoplasmic overlaps. This would be of benefit because trained technicians must screen all slides considered to be abnormal by the machine.

Selection of Glass Slides

Most of the commercially available glass microscopic slides are tapered so that one end is often 20 or more μ narrower than the other, on cross section. In addition to the taper, there are usually smaller variations in

thickness along the slide which cause difficulties in focus for an automatic scanning machine. This is a serious trouble factor for a scanning machine without an automatic focus, and would be a nuisance even for a machine with an automatic focusing device. For this reason it is important to use slides especially prepared or selected for parallelism. Slides with a maximum variation of from 2.0 to 7.5 μ [9] are being used at the present time in our laboratory.

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[9] Special Hordis Microslides. Capital Surgical Supply Company, Harrisburg, Penna.

PLATE 99

FIGURE 1.—Vaginal aspiration of Class V specimen prepared by sieve and spray technique and stained with Feulgen reaction without counterstain. × 430

FIGURE 2.—Same field as figure 1, taken at higher magnification to illustrate nuclear detail and lack of cytoplasmic stain. × 1,034

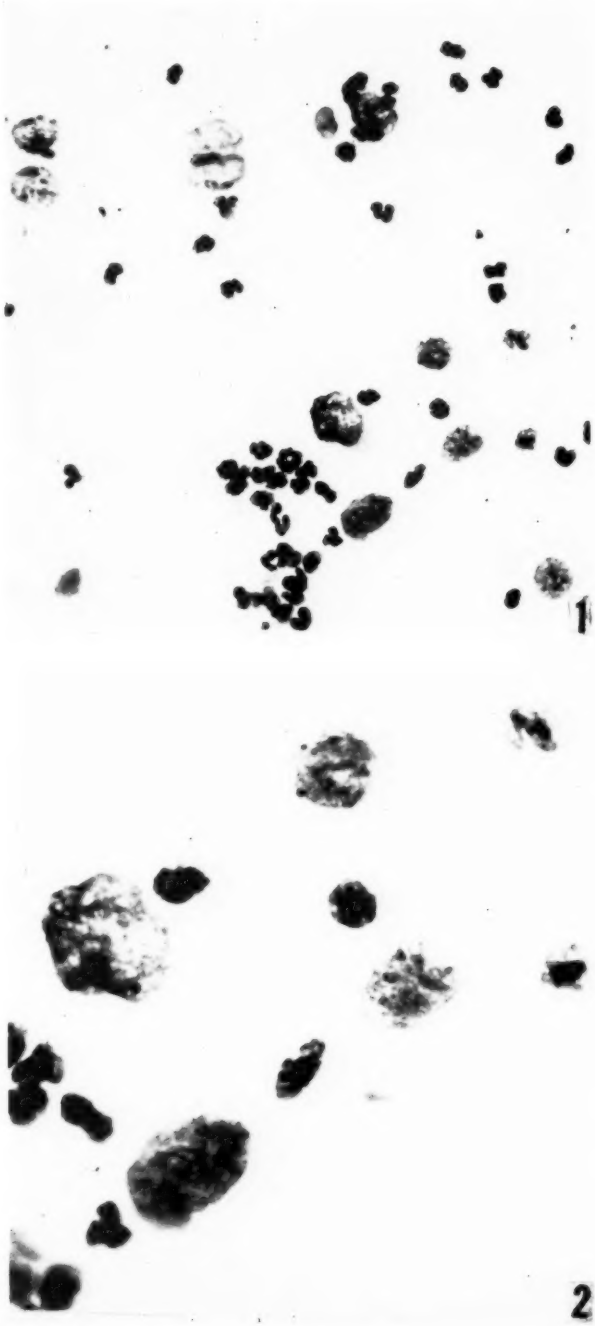

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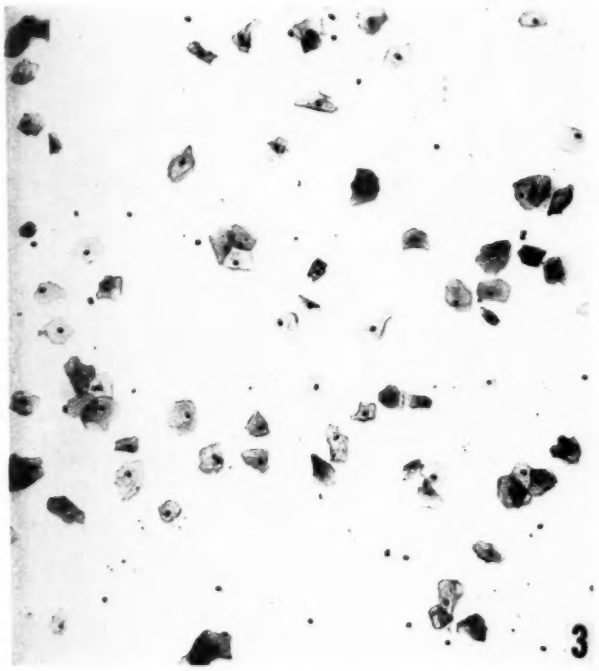
PLATE 100

FIGURE 3.—Vaginal aspiration prepared by "drop method" and stained by Papanicolaou technique to illustrate distribution of cells. × 120


3

Variations in Histologic Pattern and Functional Effects of a Transplantable Adrenal Cortical Carcinoma in Intact, Hypophysectomized, and Newborn Rats[1]

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Summary

A transplantable adrenal cortical carcinoma of the rat is described. During 7 generations of transplantation into intact, mature, male and female Osborne-Mendel rats, this tumor line, designated Adrenal cortical carcinoma 494, maintained a consistently similar histologic pattern of cords of uniform, polygonal cells. All tumor-bearing rats developed polyuria, polydipsia, and degenerative changes in the distal convoluted tubules of the kidney. They also showed atrophy of the adrenal glands, sex organs, lymphatic tissue, and pituitary gland. A subline, tumor 494H, developed after transplantation of tumor 494 into hypophysectomized rats. It differed in histologic pattern from tumor 494 in that it contained large, bizarre cells in addition to the uniform, polygonal cells of tumor 494. When tumor 494H was returned to intact, adult, male and female rats, it retained its own distinctive histologic pattern, yet it also induced polyuria, polydipsia, and renal tubular degeneration. Rats of both sexes inoculated with tumor 494H developed hyperplastic mammary glands filled with milky secretion. In the females, the uterine horns were enlarged and the vaginal epithelium mucified; in the males, the testes and accessory sex organs were extremely atrophied. A second subline, tumor 494NB, similar in histologic pattern to tumor 494H, developed when tumor 494 was transplanted to newborn rats. This subline was like subline 494H in biologic behavior, and it produced a similar effect on the organs of tumor-bearing male and female rats.—**J. Nat. Cancer Inst. 22: 1119–1155, 1959.**

Fifty-three of 59 inbred NIH Osborne-Mendel-strain rats 18 months of age or older were found to have primary adenomas or carcinomas of the cortex of the adrenal gland. Seventeen of 26 rats of this strain, 12 to 17 months of age, had hyperplastic nodules or small adenomas of the adrenal cortex. This observation is contrary to the generally accepted opinion that adrenal cortical tumors rarely occur in the rat (*1–3*). The occurrence of adrenal tumors in our Osborne-Mendel rats may be a manifestation of a general endocrine imbalance, since tumors of the adrenal gland,

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

ovary, mammary gland, or pituitary gland are often found in the same animal. It has not been determined, however, whether all the tumors of the adrenal cortex are hormonally active.

Three adrenal cortical tumors, found at necropsy of Osborne-Mendel rats over 18 months of age and diagnosed histologically as carcinoma, were transplanted. Two of the 3 tumors grew progressively, and 1 of these, Adrenal cortical carcinoma 494, has been maintained and studied in transplantation. The present paper describes the histopathology and effects of tumor 494 during 7 generations of transplantation into intact, mature rats, and the transformation both in morphology and physiologic function this tumor underwent when transplanted to hypophysectomized and to newborn rats.

Experiments and Results

Pathologic Findings in Original Tumor-Bearing Animal

Adrenal cortical carcinoma 494 was found in the right adrenal gland of a 25-month-old female inbred Osborne-Mendel rat killed and necropsied on February 2, 1955. Twenty-three months previously the rat had been inoculated intraperitoneally with a piece of gastric mucosa excised from a rat fed a diet containing catechol, for 17 months. No evidence of the transplanted gastric mucosa could be found in this rat or in any of 20 rats similarly treated. The incidence of adrenal cortical tumors was the same in the rats that received transplants of gastric mucosa as in 39 untreated rats of similar age. For these reasons, Adrenal cortical carcinoma 494 was assumed to be spontaneous in the female rat concerned here.

The right adrenal gland of the original tumor-bearing rat consisted of a soft, dark-red mass 1.0 cm. in diameter. The mass was divided; the lateral half was used for transplantation and the medial half for histologic study. The medial half was fixed and then divided into 2 parts by cutting it longitudinally in a plane perpendicular to the plane of division made at necropsy, thus supplying 2 quadrants from the original spherical mass. The freshly cut surface of each quadrant was then blocked in paraffin face down so that histologic sections cut from these blocks consisted of adjacent semicircles of tissue from the mid-portion of the medial half of the right adrenal gland. It was recognized that these sections included only a single margin immediately adjacent to the portion of tissue used for transplantation. Therefore, the paraffin blocks were melted, and the 2 quadrants reoriented and reblocked in order to obtain histologic sections from the surface closest to the tissue used for transplantation. Description *A* is based on study of the histologic sections cut from the 2 original paraffin blocks; description *B*, on study of sections cut from the reembedded tissue.

Description *A*: As shown in figure 1, sections revealed 2 histologically different tumors of the adrenal cortex (tumor #1 and tumor #2), a portion of the adrenal medulla compressed between the 2 tumors, and a rim of surviving adrenocortical tissue lying beneath the capsule.

Tumor #1 was roughly triangular, with the apex directed toward the center of the gland and the base toward the capsule. The tumor tissue was separated from the capsule by a thin layer of zona glomerulosa, and, at one point, by cells of the zona fasciculata also. Laterally, thin strands of reticulum separated the tumor from the medulla on one side and from tumor #2 on the other. Within the reticulum were enmeshed large reticuloendothelial cells containing both iron and periodic acid-Schiff-positive pigment. The cells of tumor #1 (figs. 1 and 2) were arranged in cords, from one to several cells in thickness, that were bounded by delicate strands of reticulum and collagen and were separated by blood sinuses. The cytoplasm of the tumor cells was homogeneous or finely granular and usually contained vacuoles. It was colored with both eosin and hematoxylin so that this tumor appeared darker than tumor #2. Approximately one third of the cells of tumor #1 contained cytoplasmic granules that were purplish red in the periodic acid-Schiff preparation. The nuclei were small and round and contained fine chromatin granules and from 1 to 3 nucleoli. Some cords of tumor cells contained 5 times as many nuclei as would be expected in cell cords of the same size in the normal zona fasciculata. A few tumor cells measured as much as 30 μ in diameter; their nuclei were correspondingly large and were hyperchromatic and irregular in outline. One mitotic figure was observed in every 4 or 5 high-power fields.

Tumor #2 comprised approximately two thirds of the section of the right adrenal gland available for examination (fig. 1). Except where it adjoined the medulla or the first tumor, it was separated from the capsule by a small amount of compressed adrenocortical tissue resembling the zona glomerulosa. In a few areas beneath the capsule, the zona fasciculata could be identified also. The deep aspect of tumor #2 contained large areas of hemorrhage and necrosis and widely dilated, thrombosed vessels. Parts of the tumor were composed of cords of polygonal cells that were larger and had more cytoplasm than the cells of the normal zona fasciculata (fig. 3). Occasional cords of tumor cells appeared to blend and intermingle with cells of the zona glomerulosa that were compressed against the capsule of the gland. Other parts of the tumor were composed of excessively vacuolated cells with small, deeply colored, peripherally located nuclei (fig. 4). No mitotic figures were observed in this tumor.

Description *B:* Sections from both re-embedded blocks revealed only tumor #2 and a thin rim of compressed adrenocortical tissue consisting of zona glomerulosa, and, in one area, of zona fasciculata also.

From this study we are certain that tumor #2 was contained in the portion of the adrenal gland transplanted. However, we do not know whether any other tumor was contained in that portion of the adrenal gland. Since tumor #1 did not reach the original line of division in any section examined, we are certain that we did not transplant any portion of it.

Other pathologic findings in the original tumor-bearing rat included atrophy of the left adrenal gland, 2 fibroadenomas of the mammary gland, a granulosa-cell tumor of the left ovary, a small chromophobe adenoma of

the pituitary gland, bronchiectasis, bilateral otitis media, and fatty change of the liver. The kidneys showed chronic nephritis and slight degenerative changes in the cells of the distal convoluted tubules.

The left adrenal gland was only half normal size. Cortical atrophy was pronounced (fig. 5). The layers of the cortex were poorly differentiated, although a few cords of cells, probably of the zona fasciculata, could be distinguished. The zona reticularis seemed to be the least atrophied and was the only zone containing an appreciable amount of fat, as demonstrated by Oil Red O. Within it, large reticuloendothelial cells containing both iron and periodic acid-Schiff-positive pigment were intermingled with well-defined strands of reticulum and collagen.

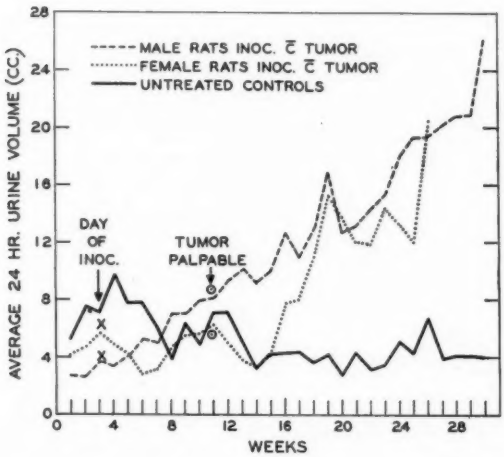
Transplantation to Intact, Adult Rats: Tumor Line 494

Pieces of the original adrenal cortical tumor were transplanted subcutaneously into 3 female rats 2 months of age. A total of 29 male and 23 female adult, intact, inbred Osborne-Mendel rats was used in the study of 7 transplant generations of tumor 494. From the outset the tumor grew successfully in all these host rats. Eight male and 8 female non-tumor-bearing rats within the same age range served as controls.

The first-generation transplant required 19 months to reach a diameter of 1.0 cm., but beginning with the second generation the transplanted tumor reached a diameter of 1.0 cm. within 1½ to 2 months after inoculation. Tumor-bearing rats from the second generation on, unless killed earlier, died, about 6 months after inoculation, from massive hemorrhage following ulceration of the tumor through the skin. Most animals were killed from 3½ to 5½ months after inoculation. The rats gained little or no weight after the transplanted tumor became palpable; terminally most of them lost weight even though their food intake was normal and water was always available. Small metastatic tumor nodules were found in the lungs and liver of a few tumor-bearing rats that survived for 5 months or more. The sex of the host did not appear to affect the growth rate, and the tumor grew as well in castrated as in intact animals. Injections of ACTH administered to a group of 8 rats during the first 6 weeks following tumor inoculation neither shortened nor lengthened the latent period.

Polydipsia and polyuria developed in all host rats about 10 weeks after inoculation (text-fig. 1). Once these symptoms appeared, increase in their severity roughly paralleled the growth of the transplanted tumor. By the time the tumors measured 3 cm. in diameter, most rats were excreting between 20 and 30 cc. of urine per day, and an occasional rat excreted as much as 60 cc. As polyuria increased, the rats drank more water, often as much as 70 cc. per day. The specific gravity of the urine decreased from an average of 1.037 to 1.012. Untreated control rats of similar age had an average urine excretion of 8.0 cc. and a water intake of approximately 36 cc. per day. Specific gravity on 24-hour urine specimens from 8 control rats varied between 1.043 and 1.031.

One female and 5 male polyuric rats bearing transplanted tumors of



TEXT-FIGURE 1.—Average daily excretion of urine from male and female rats inoculated with a fourth-generation transplant of tumor 494 compared with the excretion from control rats. At the beginning of the experiment, all rats were 6 weeks of age, and their weights were similar. Weight of control rats increased steadily from an initial average of 104 to 312 gm. at death. The average weight of tumor-bearing male rats was 118 gm. initially and 262 gm. at death; that of tumor-bearing female rats, 102 gm. initially and 172 gm. at death. Weight at death of experimental rats included weight of tumor.

Broken line: 2 male tumor-bearing rats
Dotted line: 2 female tumor-bearing rats
Solid line: 1 male and 1 female non-tumor-bearing rat

Measurement of daily urine excretion was begun when all rats were 6 weeks old; 2 weeks later the 4 experimental rats were inoculated with tumor 494. Measurement of daily urine excretion was continued until the experimental and the control rats died or were killed.

The onset of polyuria in male rats occurred 2 weeks after the transplanted tumors became palpable. In females, a sharp rise in urine excretion was noted 4 weeks after the tumors became palpable, but the urine volume did not exceed 10 cc. per day until 3 weeks later. Polyuria in tumor-bearing rats increased with growth of the transplanted tumors. Urine excretion of untreated control rats remained fairly constant and did not rise with increasing age and weight of the animals.

the fifth generation were given intramuscular injections of 0.5 cc. of pitressin tannate in oil, twice a day for 3 days. No change in the average urine excretion of the rats was noted during or following pitressin treatment.

The gross and histologic appearance of all the 52 transplanted tumors of line 494 studied in the course of 7 generations was similar (fig. 6). The tumors were all encapsulated, nodular, and well vascularized. The tumor tissue was reddish brown, soft and friable, and exuded blood from the cut surface. The central portion, especially of large tumors, was usually necrotic. Some tumors contained areas of calcification. The cells of the tumor were arranged in cords bounded by thin strands of

collagen and reticulum and separated by vascular spaces. Sometimes the tumor cells occurred in sheets. The transplanted tumors were composed of cells that were nearly all polygonal and of about the same size. The nuclei were round and basophilic, and the cytoplasm faintly eosinophilic, finely granular, and sometimes vacuolated. One or 2 mitotic figures were found in every other high-power field. Frozen sections stained with Oil Red O revealed that the cytoplasm contained fat droplets. The morphology of the tumor was consistent with a derivation from the cortex of the adrenal gland, and, taking into consideration the differences that may be manifest between a primary tumor and its transplant generations, this tumor could have been derived from tumor #2 found in the original tumor-bearing rat (figs. 3 and 4).

The kidneys of many tumor-bearing rats appeared slightly larger and softer than normal. Histologic sections revealed degenerative changes in both nuclei and cytoplasm of cells lining the distal convoluted tubules (figs. 12 and 13). This lesion was particularly well brought out by the periodic acid-Schiff reaction. In some of the distal convoluted tubules all the epithelial cells appeared degenerated; in others, some cells appeared to be normal and some degenerated, while in a few, all the cells appeared normal.

The cytoplasm of the affected cells was pale, swollen, and vacuolated. No fat, glycogen, or mucus could be demonstrated by special stains. The cytoplasm bordering the lumen appeared frayed or torn, while that near the basement membrane usually stained more deeply and contained numerous small, round granules resembling mitochondria. Linear mitochondria-like structures perpendicular to the basement membrane were noted in the cells of some distal convoluted tubules. None of these tubules was lined by the flattened type of hyperchromatic cell commonly found during regeneration after tubular damage. In some affected cells the nuclei were pyknotic or were absent. The nuclei that remained were located near the lumen in the part of the cell where the cytoplasm was torn or frayed. In some cells fine periodic acid-Schiff-positive granules were found in the cytoplasm at the luminal border. The lumens of many distal convoluted tubules contained desquamated cells and granular, eosinophilic debris.

Most of the other segments of the renal tubule showed no significant pathologic alteration. Occasionally the cells of the ascending limb of Henle's loop showed degenerative changes like those of the distal tubules. The cells of the proximal convoluted tubules appeared essentially normal, and some of them contained numerous periodic acid-Schiff-positive cytoplasmic granules (fig. 13). The lumens of a few of the proximal tubules contained cellular detritus. Most glomeruli appeared normal but, rarely, there was dilatation of capillaries and thickening of the basement membrane.

The average weight of the adrenal glands (weighed singly) of tumor-bearing rats was 6.3 mg., while that of the normal control rats was 24 mg. Both cortex and medulla of the adrenal glands of tumor-bearing rats

showed evidence of atrophy (figs. 10 and 11). In the zona fasciculata, where atrophy was greatest, the usual cordlike arrangement of cells was lost, and the sinusoids were dilated. The zona glomerulosa was somewhat thinner than normal, and its characteristic glomerular pattern was not evident. Frozen sections stained with Oil Red O revealed virtually no fat in the zona fasciculata and zona glomerulosa, and very little fat in the zona reticularis. Numerous large reticuloendothelial cells containing iron, a small amount of fat, and a few periodic acid-Schiff-positive granules were found in the zona reticularis and the inner part of the zona fasciculata. Few, or none, of these cells were seen in the adrenal glands of untreated rats.

The adrenal medulla of the tumor-bearing rats showed changes in the cells and varying degrees of congestion. About half the medullary cells appeared normal except for an abnormally large amount of pigment (fig. 11). Other cells were small and polygonal, and groups of them were closely fitted together. Usually the outlines of these abnormal cells were distinct, but occasionally a group of cells resembled a syncytial multinucleated cytoplasmic mass. The cytoplasm of the small abnormal cells was free of fat. It was colored purplish brown by the periodic acid-Schiff reaction and a diffuse brown in hematoxylin and eosin preparations. The nuclei, which were colored an even deeper brown, were round or oval and had a thick nuclear membrane that enclosed both fine and coarse chromatin granules and usually 1 nucleolus.

The gonads and accessory sex organs of both male and female tumor-bearing rats consistently showed atrophy of epithelial cells and stroma (figs. 14 to 19). On the average, the testis and uterus weighed half as much, and the ovary, one third as much, as the corresponding organs of non-tumor-bearing rats. In no tumor-bearing rat did the paired seminal vesicles weigh more than 200 mg., while in control rats the average weight was 1275 mg.

Often the size of the testis decreased with the increasing age of the transplanted tumor. Arrest of spermatogensis at the spermatid stage was commonly observed, although histologic alterations varied from diminished spermatogenesis in the least affected tubules to degeneration of spermatocytes and of Sertoli cells in those most affected (figs. 14 to 16). The size and number of interstitial cells were usually decreased. The decreased size of the seminal vesicles was explained by the absence of the usual fluid within the lumen and by the shrinkage of the epithelial lining cells.

The uterine horns of tumor-bearing rats were pale, thin, elongated, and fibrous. The endometrium, endometrial stroma, and myometrium were all atrophied (figs. 18 and 19). The vaginal epithelium resembled that of a spayed rat, being even thinner and showing less secretory activity than that of a normal rat in mid-dioestrus (fig. 22). Many of the ovarian follicles were atretic. Corpora lutea were usually present and appeared normal or only slightly atrophic, but in a few rats inoculated with tumor 494 at 4 weeks of age, corpora lutea were not found. The amount of

stroma in the ovaries was diminished, but stromal fat content was about the same as in untreated controls. Nests of interstitial cells with a radiating spokelike arrangement of the nuclear chromatin were found in many ovaries (fig. 24). These were considered to be the type of "wheel cell" observed in the ovaries of hypophysectomized rats (*4*) and in the ovaries of old rats with disorders of the estrous cycle (*5*).

The pituitary glands of rats that had borne the transplanted tumor 494 for 3 months or less did not differ in gross appearance or in average weight from the pituitary glands of matched controls. Histologic sections stained by Mallory's acid fuchsin-aniline blue method revealed numerous, large, vacuolated, partially degranulated basophils in the anterior lobe. These were considered to be castration cells. No change was noted in the acidophils or chromophobes.

In rats that had borne the transplanted tumor 494 for more than 3 months, the pituitary glands weighed on the average a little less than the glands of the matched controls. Blood spaces occupied more of the anterior lobe, and the cells were more compact and smaller than in the controls. The acidophils, in addition to being smaller and having less cytoplasm than the acidophils in the controls, usually had small hyperchromatic nuclei; some nuclei showed pyknosis, karyolysis, and karyorrhexis. Although no cell counts were made, study of serial sections gave the impression that the total number of basophils was decreased and that the vacuolated basophils were absent or could be found less frequently than in rats killed within the first 3 months after inoculation with the tumor.

Histologic sections of the mammary glands revealed normal development of ducts in the females and of acini in the males. In some of the tumor-bearing rats of both sexes the mammary-gland tissue resembled that occasionally seen in old animals in that the ducts and acini were shrunken and surrounded by thick, hyalinized, connective tissue (figs. 27 and 31). Compare with normal glands (figs. 26 and 30). Mammary ducts were more prominent than normal in several tumor-bearing males that had greatly atrophied testes, while acini were the predominant structure in female rats with the greatest ovarian atrophy, or in which corpora lutea were absent.

Atrophy of the thymus gland (fig. 25) and lymphoid follicles of the intestine was evident in all tumor-bearing rats. In some rats hematopoiesis and pigment deposition in the splenic pulp was decreased, and the splenic follicles appeared to be smaller than normal; in others, no abnormalities of the spleen could be detected. Leukocyte counts ranged from 2,450 to 4,100 per $mm.^3$ in tumor-bearing rats and from 7,350 to 8,300 per $mm.^3$ in the controls. Erythrocyte counts ranged from 10,530,000 to 12,050,000 per $mm.^3$ in tumor-bearing rats and from 7,170,000 to 8,200,000 per $mm.^3$ in the controls. No comparison of eosinophil counts was made. The thyroid gland, salivary glands, pancreas, and other organs of rats bearing the transplanted tumor 494 showed no histopathologic alterations attributable to the experimental procedure.

Transplantation to Hypophysectomized Rats and Development of Tumor Line 494H

Growth for 1 generation in a hypophysectomized rat induced an unexpected transformation in tumor 494—a transformation manifested both in the histologic pattern and in the physiologic effect of the tumor. Altered tumor line 494H was derived as follows: A fourth-generation tumor of line 494 grown in a mature, intact, female rat was transplanted to 6 hypophysectomized rats, in May, 1957. The transplanted tumor reached a diameter of 0.5 cm. in 1 male and in 1 female hypophysectomized rat after a latent period of 7 months; it did not grow at all in the other 4 rats. Intact rats inoculated at the same time developed tumors that measured 1.0 cm. in diameter within 2 months; these tumors in their subsequent growth and in their effect upon the hosts did not differ from other tumors of line 494.

Sections of the transplanted tumors of line 494H that grew in both the hypophysectomized rats contained 2 types of cell (fig. 7). One was the uniform polygonal cell that comprised the entire cell population of unaltered line 494. The other type of cell was much larger, with deeply colored, vacuolated cytoplasm, and irregularly shaped, hyperchromatic nuclei. Mitotic figures were exceedingly numerous. Aside from the alterations secondary to hypophysectomy, no other gross or histologic changes attributable to the effect of the transplanted tumor, 494H, were observed in the host rats. Serial sections of the sella turcica revealed a tiny fragment of anterior pituitary-gland tissue in the male rat, but none in the female rat.

Pieces of tumor 494H that had grown in the hypophysectomized female rat were transplanted subcutaneously into 2 intact male and 2 intact female rats 3 months of age. These 4 animals were not litter mates. In all of them the tumor grew readily and reached a diameter of 1.0 cm. within 2 months. Shortly thereafter, the host rats developed polyuria and polydipsia. They were killed from 5 to 6 months after inoculation. All 4 transplanted tumors were composed of small polygonal cells and large bizarre cells, and thus resembled the tumor grown in the hypophysectomized rat from which they had been transplanted (fig. 7). A similar combination of cells comprised metastatic tumor tissue in the lungs of all 4 rats and in the adrenal glands of the 2 females (fig. 9). The kidneys revealed the degenerative lesion of the distal convoluted tubules.

Other effects of tumor 494H were decidedly different from the effects of tumor 494 that had always been carried in rats with intact pituitary glands. The adrenal glands of the animals bearing tumor 494H were only moderately atrophied, and the zona glomerulosa was the only layer of the cortex markedly depleted of fat. Mammary glands of both male and female rats were filled with milky secretion (figs. 28 and 32). Atrophy of the testes was extreme, and spermatogenesis was arrested at the stage of the primary spermatocyte (fig. 17). Atretic follicles but no corpora lutea were present in the ovaries. The uterine horns were enlarged (fig.

20). The columnar endometrial cells were unusually tall, and their cytoplasm contained occasional droplets of mucus; the nuclei were basally situated. An extraordinary degree of mucification was present in the intermediate layer of the vaginal epithelium (fig. 23).

Transplantation to Newborn Rats and Development of Tumor Line 494NB

Altering the environment of tumor 494 by transplanting it to newborn rats induced a transformation in its histologic pattern similar to that observed when tumor 494 was grown in hypophysectomized rats. This altered line, designated 494NB, was derived from a fifth-generation tumor of line 494 grown in a mature, intact, male rat. Approximately 1 gm. of tumor tissue was homogenized with 10 cc. of physiologic saline, and 0.1 cc. of this brei was injected subcutaneously into 5 female and 3 male newborn littermate rats less than 24 hours old. Transplanted tumor 494NB required 11 weeks (compared with 8 weeks for tumor 494) to reach a diameter of 1.0 cm., but from then on tumor 494NB grew rapidly. Polyuria and polydipsia were first manifest when the rats were 3 months old. The 8 animals were killed from 4 to 4½ months after inoculation.

The transplanted tumors of line 494NB were composed in part of the uniform type of polygonal cell characteristic of tumor 494, but they also contained the large, irregular, bizarre cells with hyperchromatic nuclei and many mitotic figures (fig. 8) as seen in line 494H (fig. 7). Metastatic tumor tissue containing both small polygonal cells and large bizarre cells was found in the liver and lungs of all 8 rats bearing tumor 494NB, in the adrenal glands of 2 males and 3 females, in the spleen of 1 female, and in the mediastinal lymph nodes of 1 male. The mammary glands (figs. 29 and 33), testes, ovary, uterus (fig. 21), and vagina (fig. 23) resembled, in gross appearance and in histology, the corresponding organs of rats bearing transplants of tumor 494H. Degenerative changes in the distal convoluted tubules of the kidney were of the same degree as previously described in rats bearing tumor lines 494 and 494H.

Discussion

Comparison of tumor 494 with other adrenal cortical tumors of the rat.—Primary tumor 494 resembled in some respects the tumors that Houssay and his associates induced in the adrenal glands of rats by castration (*1*, *6*). These authors classified the cells of their tumors as A, B, and C. Cells of Type A were small and similar to cells of the zona glomerulosa, and in the early stages of tumor growth they formed triangular subcapsular nodules. Cells of Type B were polygonal and larger than Type A; the cytoplasm was finely granular and vacuolated, and the cells were arranged in nodules or cords reminiscent of the zona fasciculata. Cells of Type C were large, the nuclei were peripherally placed, and the cytoplasm was extensively vacuolated and contained both fat and periodic acid-Schiff-positive droplets. The Type C cell of the tumor described by Houssay *et al.* resembled the cells of tumor #2 (figs. 3 and 4) found in the

adrenal gland of our original tumor-bearing rat. Cell types A and B were similar to some of the smaller, more deeply colored, cells of our tumor #1 (fig. 2). The adrenal cortical tumors of castrated rats described by Houssay and his coworkers were often associated with tumors of the pituitary gland and mammary gland, which has been our experience with the spontaneous tumors of Osborne-Mendel rats.

The transplanted tumor of line 494 is similar in histologic appearance to several other transplantable adrenal cortical tumors of the rat (*2*, *3*, *7*)—in its effect on the host it is similar in some respects and different in others. A tumor that originated in an Osborne-Mendel rat fed a low protein diet was successfully transplanted by Mulay and Eyestone (*7*) to untreated male and female rats. This tumor induced atrophy of the adrenal glands of the host, but no striking changes in any other endocrine gland and no measurements of the urine output of tumor-bearing rats were reported by these investigators. It is not known whether this tumor's functional capacity was the same as that of tumor 494.

In its physiologic effects, including the induction of polyuria and polydipsia, tumor 494 resembles the adrenal cortical tumor of WR rats described by Cohen, Furth, and Buffett (*2*). These authors, however, did not mention a renal lesion as being associated with the tumor they studied. When Cohen *et al.* (*8*) incubated slices of rat adrenal tumor with and without ACTH, they were able to demonstrate that its secretion consisted mainly of corticosterone. No bioassay has been made on tumor 494, but is seems reasonable to postulate that it, too, may secrete adrenal corticoids.

Tumor 494 grows well in all untreated mature rats and in castrated rats of both sexes. In this respect it differs from the tumor described by Cohen and his associates, which grows better in males than in females and is at least partially dependent upon hormonal imbalance produced in the host by gonadectomy, adrenalectomy, or thyroidectomy. The growth pattern of tumor 494 also differs from that of an adrenal cortical tumor in A × C rats, described by Iglesias and Mardones (*3*, *9*), which grows better in females than in males, requires no induced hormonal imbalance for its growth, and is partially inhibited by ACTH. ACTH neither retarded nor accelerated the growth of our tumor 494, and its functional, biologic, and histologic characteristics were not altered by administering ACTH to the hosts.

Association of polyuria with degenerative changes in the distal convoluted tubules.—Several investigators (*10–12*) after studying the physiologic effects of various adrenal steroid hormones on the kidney have postulated that these hormones damage the cells of the distal convoluted tubules, thus impeding the normal reabsorption of water and producing polyuria. However, none of these investigators described a histologic lesion of the kidney. In the present study we have demonstrated that the polyuria and polydipsia observed in rats bearing tumor 494 and its sublines are associated with a morphologic lesion of the distal convoluted tubules of the kidney. Slight degenerative changes in the cells of the

distal convoluted tubules were observed in a few rats killed before the onset of polyuria and polydipsia, and the most marked degeneration was found in rats that exhibited these symptoms in greatest severity for the longest period of time. It is therefore probable that secretions from the transplanted tumor induced the tubular degeneration. Whether, once initiated, the constant and increased urine flow may also have played a part in the degenerative renal process is not known. The total daily output of urine was not influenced by treatment of the rats with pitressin; hence, it seems probable that the polyuria had a renal origin rather than a hypothalamic or posterior pituitary origin.

Comparison of the renal lesion of rats bearing tumor 494 with the renal lesion of potassium deficiency.—The kidneys of rats bearing the transplanted adrenal tumor resembled those of rats fed a potassium-deficient diet only in that both manifested increased weight, dilatation of the distal convoluted tubules, and vacuolization of the tubular cells. In other respects the two lesions differed. Neither the cellular regeneration nor the lipoidal droplets and calcareous material in the epithelial cells of the distal convoluted tubules, described by Follis, Orent-Keiles, and McCollum (*13*) as characteristic of potassium deficiency, were present in the kidneys of our tumor-bearing rats. The renal degeneration associated with tumor 494 was limited almost exclusively to the distal convoluted tubule, whereas the nephron in its entirety was involved in the lesion that Tauxe and his associates (*14*) induced in rats by a potassium-deficient diet. We did not observe the retrogressive alterations or the prolific regenerative hyperplasia of the cells of both collecting and proximal convoluted tubules described by Oliver and his coworkers (*15*) during the early stages of potassium depletion. Therefore, the morphology of the renal lesion in our tumor-bearing rats differs from that of rats with a potassium deficiency.

Comparison of tumor 494 with tumor 494H.—In all 7 generations of line 494, the subcutaneous transplants and the few pulmonary and hepatic metastases had the same histologic pattern of cords of uniform polygonal cells (fig. 6). The functional effects of the tumors upon the hosts were also similar. It seems probable that the tumors secreted an excess of certain hormones normally secreted by the cortex of the adrenal gland and that these tumor secretions, by suppressing the pituitary gland, indirectly induced atrophy of the adrenal glands, sex organs, and lymphoid tissue. Renal damage, polyuria, and polydipsia may have been caused by secretions from the transplanted tumor acting directly upon the kidney.

The tumors of subline 494H, developed by sojourn of tumor 494 for 1 generation in a hypophysectomized rat and subsequently returned to intact, mature, male and female rats, were all alike in biologic behavior, histologic pattern, and physiologic effect, but different in some respects from tumor 494. Tumor 494H consisted, in part, of cords of uniform polygonal cells and, in part, of cells that were bizarre in shape, variable in size, and usually contained 1 large hyperchromatic nucleus (fig. 7). A similar combination of cells was found in frequent, large, metastatic

tumors in the lungs and adrenal glands (fig. 9). The appearance of the mammary glands, pituitary glands, testes, ovaries, uteri, vaginas, and adrenal glands of intact, mature rats bearing tumor 494H was similar to that observed after the administration of estrogen and progesterone. Polyuria, polydipsia, and degenerative changes in the renal tubules indicate that the altered tumor 494H retained at least part of the functional activity of tumor 494.

Change in environment of the tumor cells during their growth is the most plausible explanation for the difference between tumor 494 and tumor 494H, since the same tumor of line 494 that was used for transplantation into the hypophysectomized rats was also transplanted into intact, mature rats. In these latter rats neither the tumor cells nor their effects upon the hosts were altered in any respect. Why transplantation in a hypophysectomized rat should bring about the alterations observed in tumor 494H, however, is not clear. The concomitant appearance in tumor 494H of a type of cell and of a functional effect not hitherto associated with any tumor of line 494 makes it reasonable to assume that the new cell and the new functional activity are related.

The question then arises as to the source of the altered cells. One possibility is that they developed from the uniform polygonal cells and thus represent a change toward anaplasia, a common development in transplantable tumors. However, when the cells of a tumor become anaplastic, the tumor nearly always loses at least some of its functional capacity. We cannot recall any manifestation by tumor cells of a definitely new functional activity coincident with morphologic anaplasia. Another possible explanation for the altered cells in tumor 494H is that they represent latent cells, which were transplanted from the original tumor and suppressed in the intact animal by some restraining influence of the normal pituitary gland. The absence of the pituitary gland may have allowed some of these tumors cells to grow without restraint and to manifest a hitherto unexpressed hormonal activity. Since the large, bizarre cells were observed for the first time in tumor 494H, they may well be the cells associated with the altered function.

Comparison of tumors 494, 494H, and 494NB.—The tumors of subline 494NB, which developed when the tumor of line 494 was transplanted into newborn rats, contained large, bizarre cells as well as the smaller polygonal cells characteristic of tumor 494. Tumor 494NB thus resembled tumor 494H in histologic pattern, since the altered, bizarre cells comprised approximately half the cell population of both tumors. These sublines were also similar in functional effects because both produced the same changes in the sex organs and mammary glands. Tumor 494H and 494NB were alike in that all tumors of both lines metastasized. The metastases occurred earlier and in more sites than in any rat bearing tumor 494. Tumor 494 metastasized infrequently to the liver and lungs of rats that had borne transplants for a long period. Polyuria, polydipsia, and renal tubular degeneration were observed in rats bearing tumor 494 and in rats bearing the sublines 494H and 494NB.

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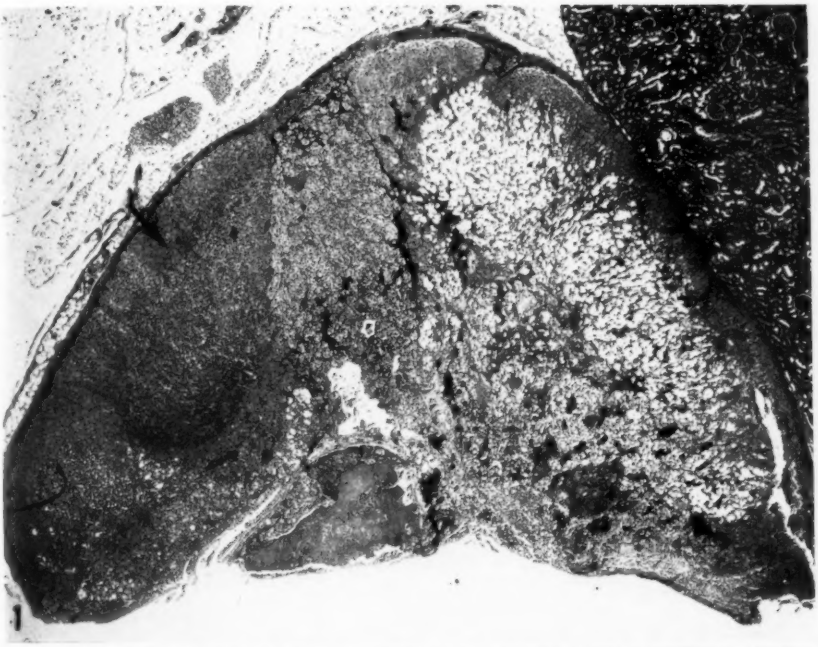
PLATES

PLATE 101

FIGURE 1.—Half of the right adrenal gland of original tumor-bearing rat. Line of incision dividing the adrenal gland into 2 parts at necropsy is at *bottom* of illustration. Elsewhere the gland is surrounded by the capsule, beneath which lies a thin rim of compressed adrenocortical tissue. *Arrow* points to tumor designated #1 in the text; the compressed medulla is just to *right*. All the rest of the section contains tumor #2, which extends to the line of incision and was therefore included in the tissue used for transplantation. *Note* the dilated, thrombosed vessel along the line of incision. A portion of the kidney is at *right* margin of the illustration. Hematoxylin and eosin. × 22

FIGURE 2.—Tumor #1 of the right adrenal gland of original tumor-bearing rat. Hematoxylin and eosin. × 360



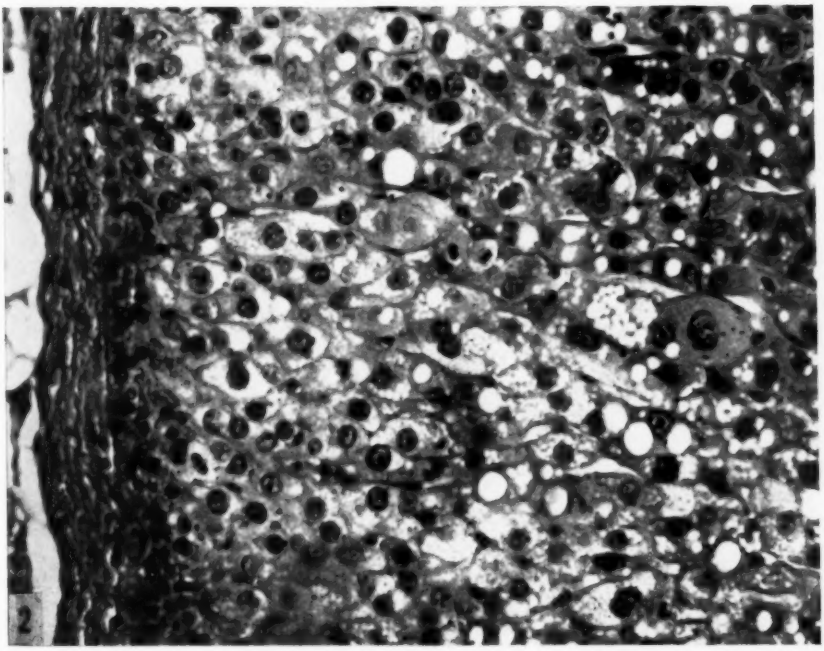
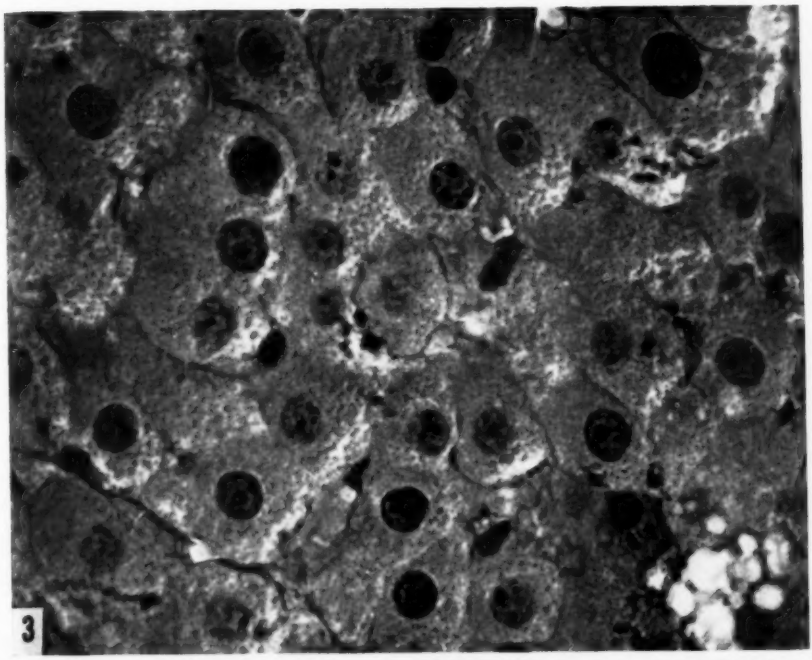


PLATE 102

FIGURE 3.—Tumor #2 of the right adrenal gland of original tumor-bearing rat. Hematoxylin and eosin. × 1,050

FIGURE 4.—Tumor #2 of the right adrenal gland of original tumor-bearing rat: Cells show excessively vacuolated cytoplasm and peripherally located nuclei in contrast to the cells of the portion of the tumor illustrated in figure 3. Hematoxylin and eosin. × 1,050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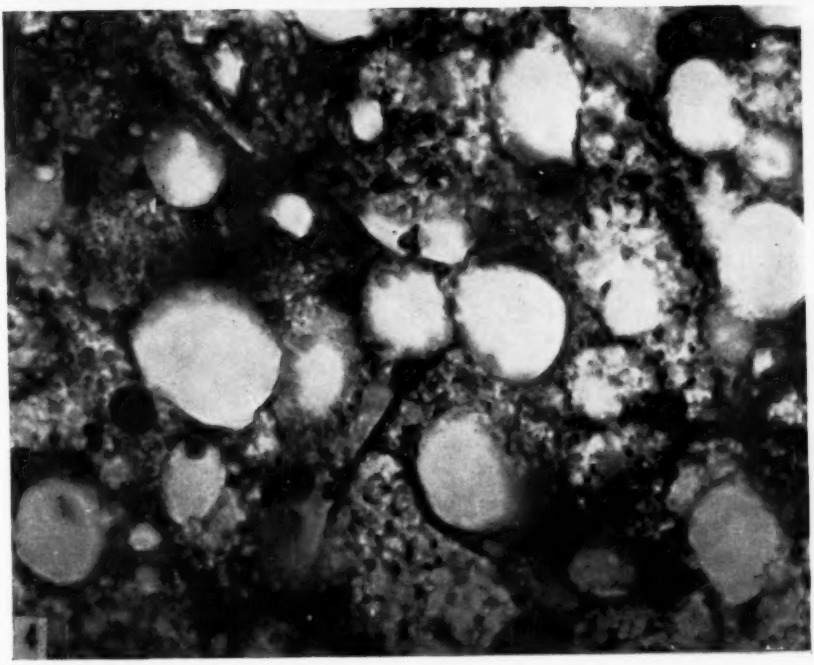
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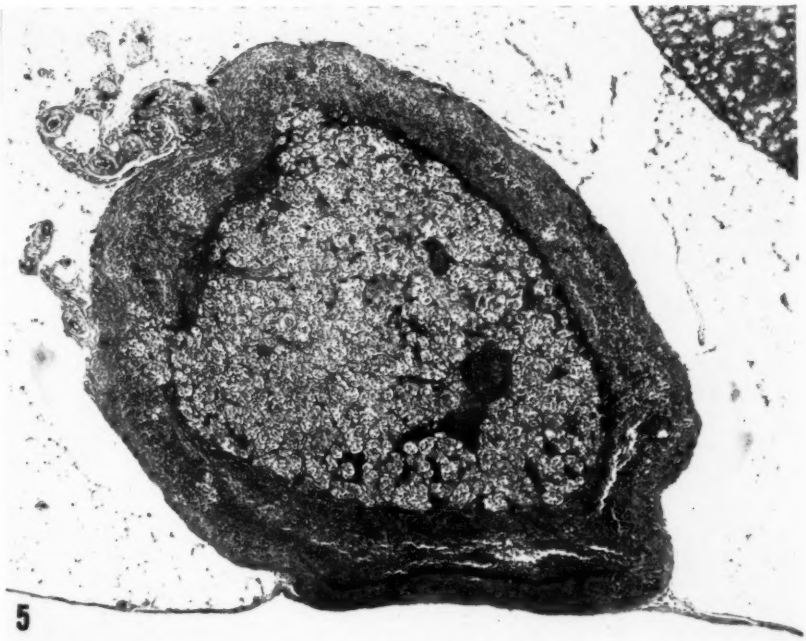
PLATE 103

FIGURE 5.—Left adrenal gland of original tumor-bearing rat, showing pronounced atrophy of the cortex. Hematoxylin and eosin. × 50

FIGURE 6.—Tumor 494. A fifth-generation transplant grown in the subcutaneous tissues of a 6-month-old intact female rat inoculated 4½ months previously. *Note* uniform polygonal cells in cordlike arrangement and large, thin-walled vascular channels. Hematoxylin and eosin. × 210



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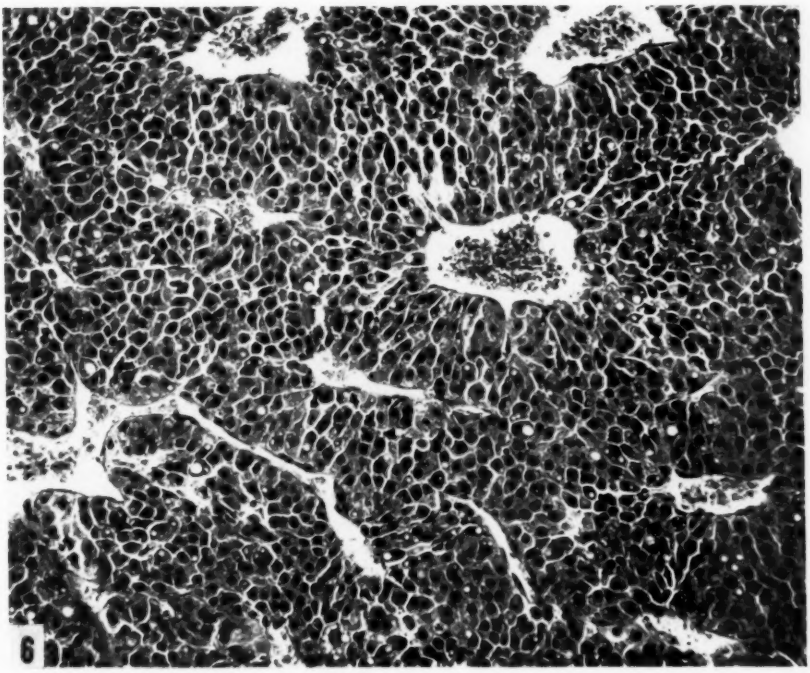

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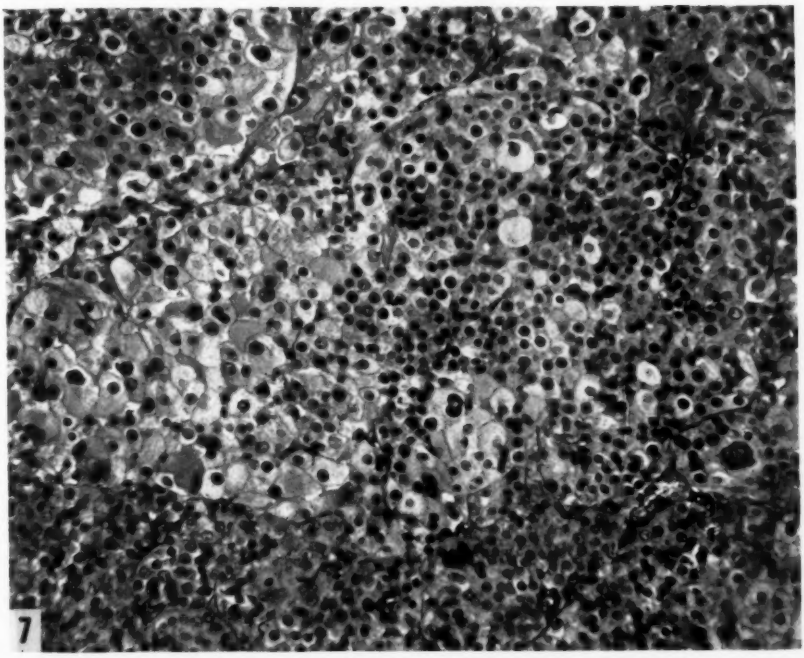
PLATE 104

FIGURE 7.—Tumor 494H. Subcutaneous transplant tumor from an 8-month-old intact female rat inoculated 5 months previously with tumor 494H that had been grown in a hypophysectomized female rat. *Contrast* histologic appearance with that of the corresponding generation of tumor 494 shown in figure 6. The large, bizarre cells that first appeared in tumor 494H can be correlated with a change in the functional effects of the transplanted tumor. Hematoxylin and eosin. × 210

FIGURE 8.—Tumor 494NB. Subcutaneous transplant tumor from a 4-month-old female rat inoculated, when less than 24 hours old, with the fifth-generation transplant of tumor 494 grown in an intact mature male rat. The histologic pattern of line 494NB is like that of line 494H (fig. 7) and different from that of tumor 494 (fig. 6). Hematoxylin and eosin. × 210



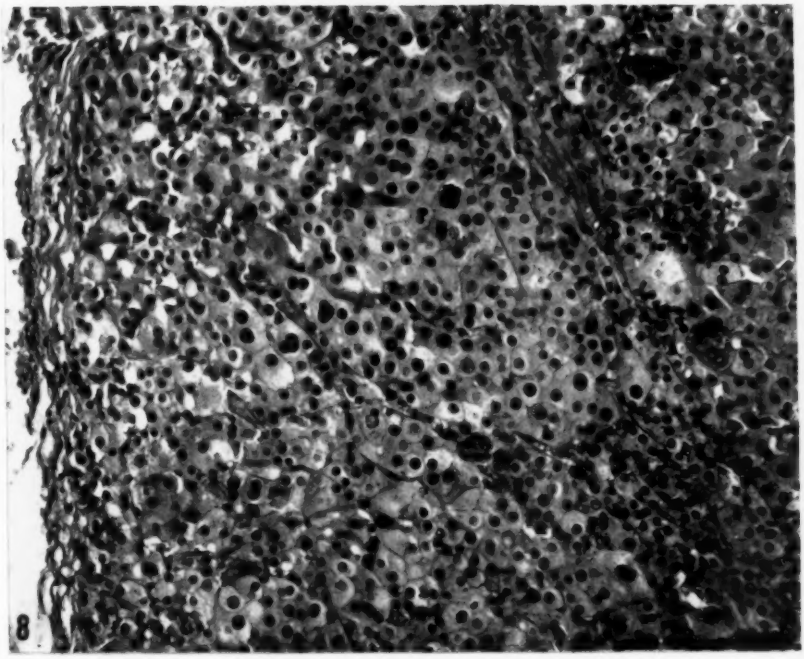


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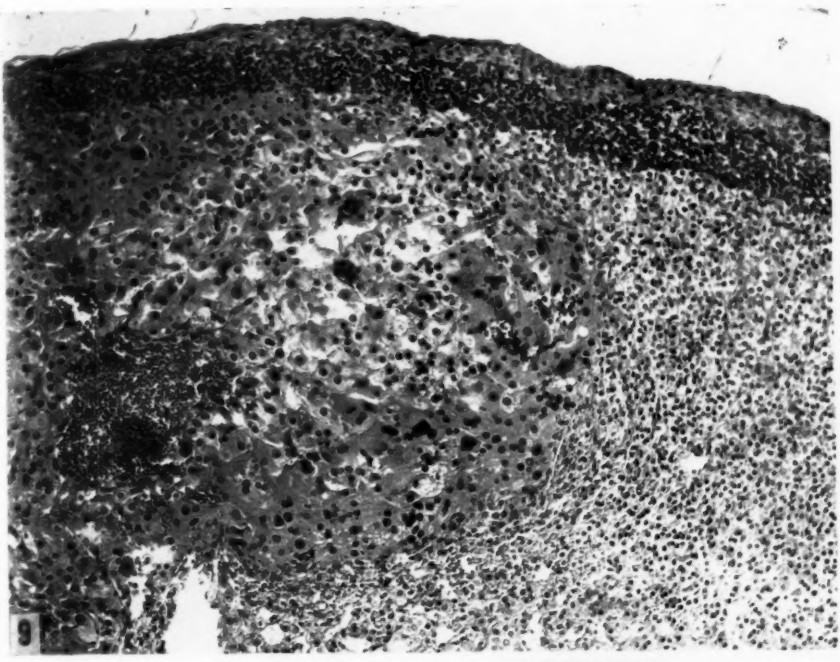
PLATE 105

FIGURE 9.—Metastasis of tumor 494H in the adrenal gland of an intact female rat, 8 months of age, inoculated 5 months previously with the tumor grown in the hypophysectomized female rat. Large, bizarre cells are like those illustrated in figure 7. The metastatic tumor has distorted this portion of the adrenal gland so that the cortical atrophy cannot be appreciated. Hematoxylin and eosin. × 135

FIGURE 10.—Adrenal gland of an untreated, non-tumor-bearing female rat 4½ months of age. Hematoxylin and eosin. × 135

FIGURE 11.—Adrenal gland of a 4½-month-old female rat inoculated 3 months previously with Adrenal cortical carcinoma 494. The cortex is atrophied, and the medulla contains small, deeply colored cells. Hematoxylin and eosin. × 135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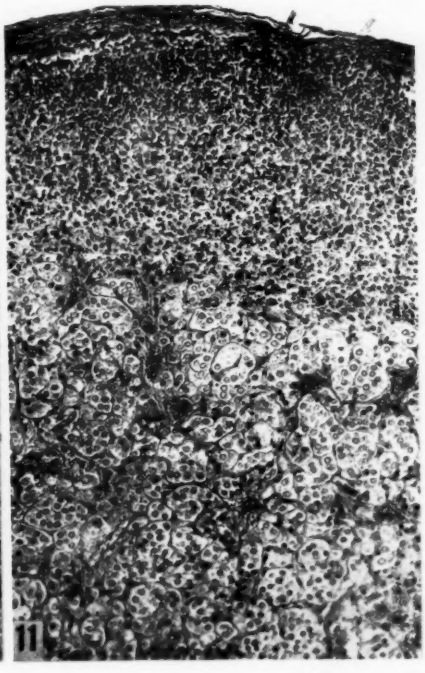
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PLATE 106

FIGURE 12.—Kidney of a 7½-month-old female rat inoculated 4½ months previously with the fourth-generation transplant of tumor 494. The subcutaneous transplant tumor weighed 10.5 gm. Degenerative changes are present in the cells of the distal convoluted tubules. In the week before necropsy the rat's average daily water intake was 57 cc. and its average daily urine excretion was 24 cc. Periodic acid-Schiff. × 260

FIGURE 13.—Kidney of a 9-month-old male rat inoculated 5½ months previously with the fourth-generation transplant of tumor 494, showing a higher-power view of the degenerated cells of the distal convoluted tubules. Many cells of the normal proximal convoluted tubules contain periodic acid-Schiff-positive granules. In the week before necropsy this rat had an average daily urine excretion of 60 cc., the largest daily output of any rat in our series. Periodic acid-Schiff. × 470

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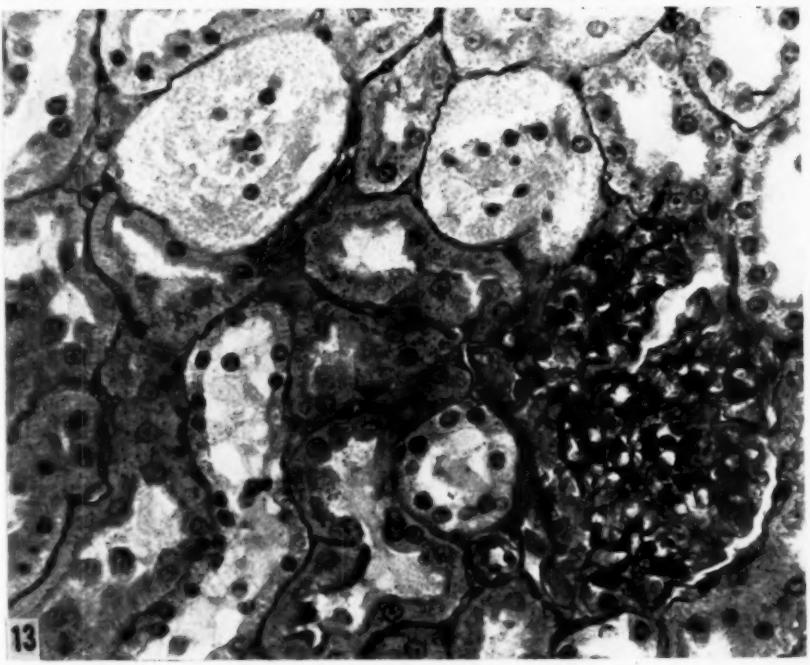
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PLATE 107

FIGURE 14.—Testis (weight 1332 mg.) from a normal untreated rat 8 months of age. Hematoxylin and eosin. × 165

FIGURE 15.—Testis (weight 675 mg.), showing moderate atrophy, from a 9-month-old rat inoculated 5½ months previously with tumor 494. Hematoxylin and eosin. × 135

FIGURE 16.—Testis (weight 381 mg.), showing pronounced atrophy, from an 8½-month-old rat inoculated 5½ months previously with tumor 494. There is no evidence of spermatogenesis, and only Sertoli cells remain in the tubules. Hematoxylin and eosin. × 135

FIGURE 17.—Testis (weight 215 mg.), showing pronounced atrophy, from an 8-month-old rat inoculated 5 months previously with tumor 494H. There is interstitial edema and pronounced atrophy of tubular cells. Spermatogenesis is arrested at the stage of the primary spermatocyte. *Contrast* with lesion illustrated in figure 16. Hematoxylin and eosin. × 135

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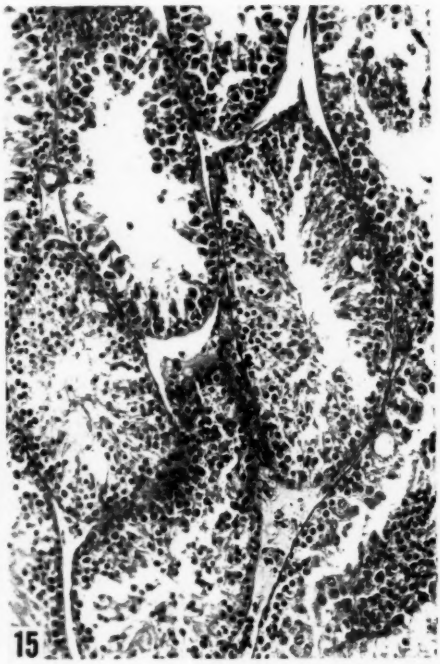
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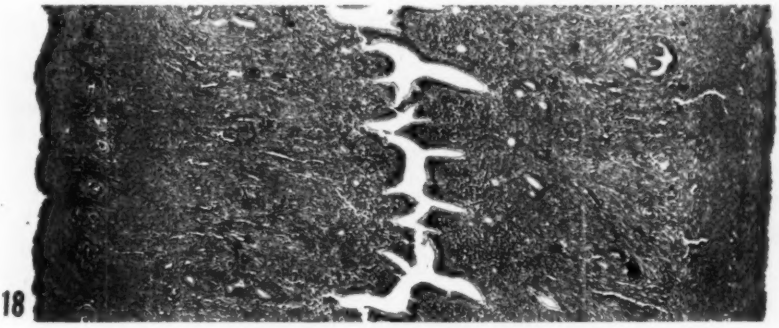
PLATE 108

FIGURE 18.—Uterus (weight 323 mg.) from a normal rat 6 months of age. Hematoxylin and eosin. × 95

FIGURE 19.—Atrophic uterus (weight 109 mg.) from a 4½-month-old rat inoculated 3 months previously with a third-generation transplant of tumor 494. Hematoxylin and eosin. × 95

FIGURE 20.—Enlarged uterus (weight 502 mg.) from a 9-month-old rat inoculated 6 months previously with tumor 494H. Compared with the normal uterus (fig. 18) this uterus shows hypertrophy and hyperplasia of endometrium, endometrial stroma, and myometrium. Hematoxylin and eosin. × 95

FIGURE 21.—Enlarged uterus (not weighed) from a rat bearing tumor 494NB. The tall hyperplastic epithelial cells are like those illustrated in figure 20. Hematoxylin and eosin. × 200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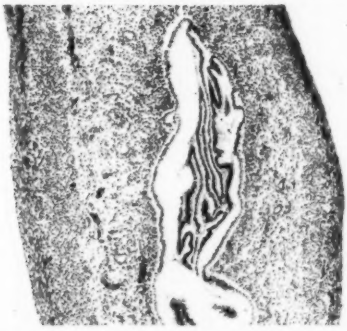
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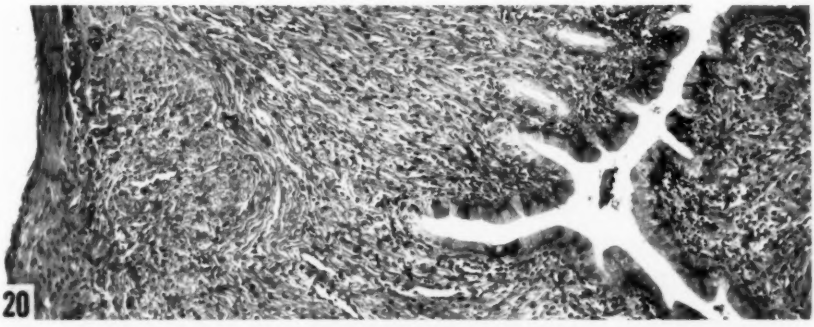
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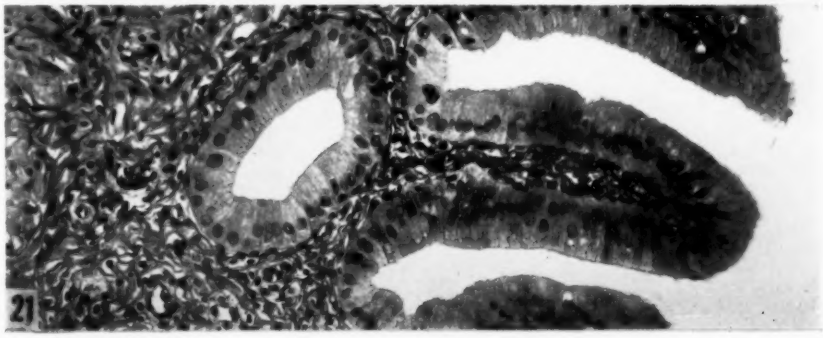
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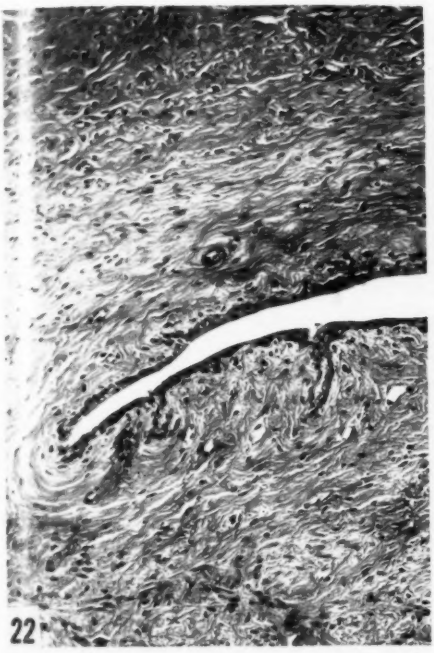
PLATE 109

FIGURE 22.—Vagina from a 4½-month-old rat inoculated 3 months previously with tumor 494, showing atrophy of the mucosa and fibrosis of the wall. Hematoxylin and eosin. × 170

FIGURE 23.—Vagina from a 4-month-old rat bearing tumor 494NB. The epithelium is thickened and shows mucification. The underlying muscular wall is not included in this illustration because it is separated from the subepithelial connective tissue by a zone of edema shown at *bottom* of illustration. Hematoxylin and eosin. × 170

FIGURE 24.—Wheel cells in the ovary of a 4½-month-old rat inoculated 3 months previously with the sixth-generation transplant of tumor 494. Periodic acid-Schiff. × 1,050

FIGURE 25.—Atrophic thymus gland of an 8-month-old female rat inoculated 4½ months previously with the fifth-generation transplant of tumor 494. Hematoxylin and eosin. × 125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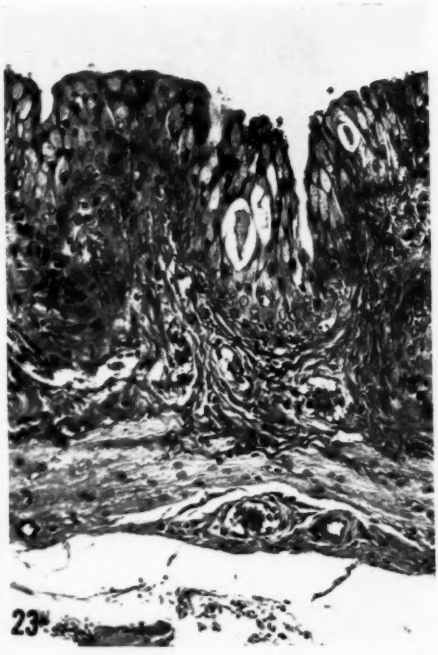
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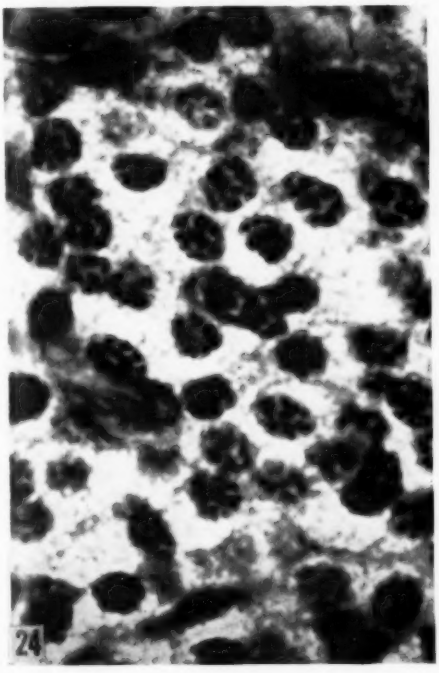
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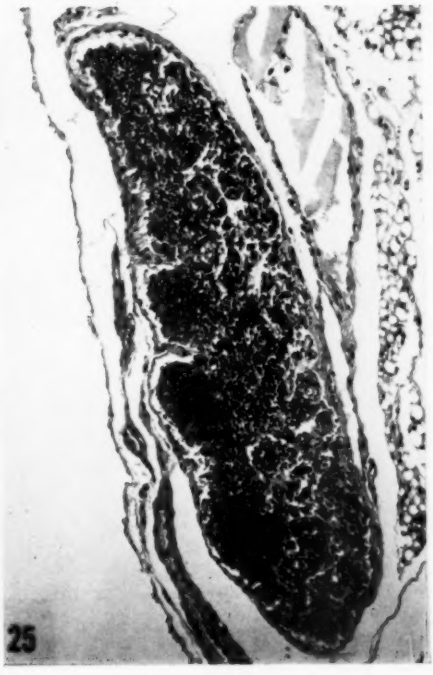
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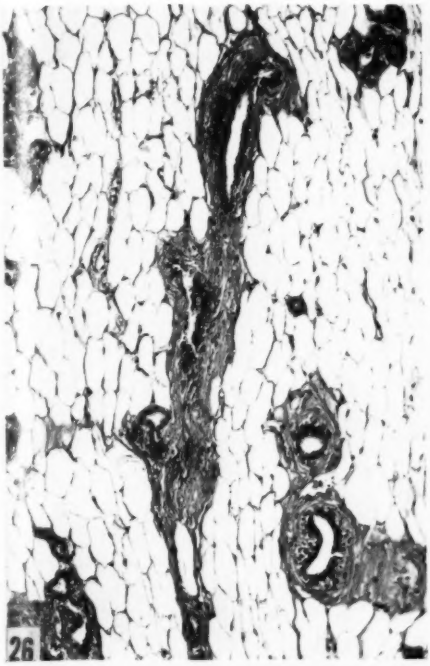
PLATE 110

FIGURE 26.—Mammary gland of a normal female rat 8 months of age. Hematoxylin and eosin. × 125

FIGURE 27.—Mammary gland of an 8-month-old female rat inoculated 4½ months previously with tumor 494. There is an excessive amount of hyalinized stroma around the ducts. Hematoxylin and eosin. × 125

FIGURE 28.—Mammary gland of a 9-month-old female rat inoculated 6 months previously with tumor 494H. The ducts and acini are hyperplastic and contain a milklike secretion. Hematoxylin and eosin. × 125

FIGURE 29.—Mammary gland of a 4-month-old female rat bearing tumor 494NB. The ducts and acini are hyperplastic and contain a milklike secretion. Hematoxylin and eosin. × 125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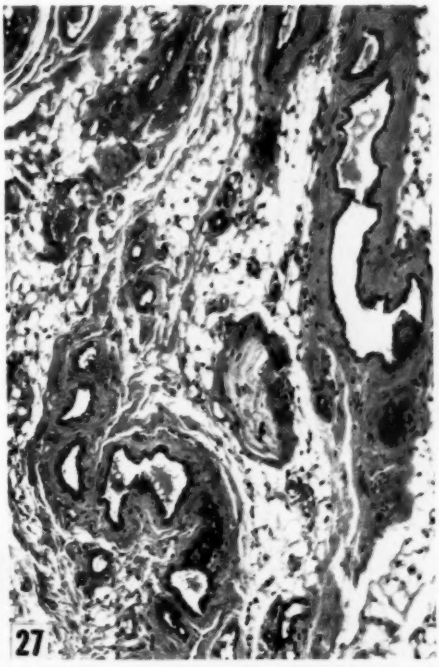
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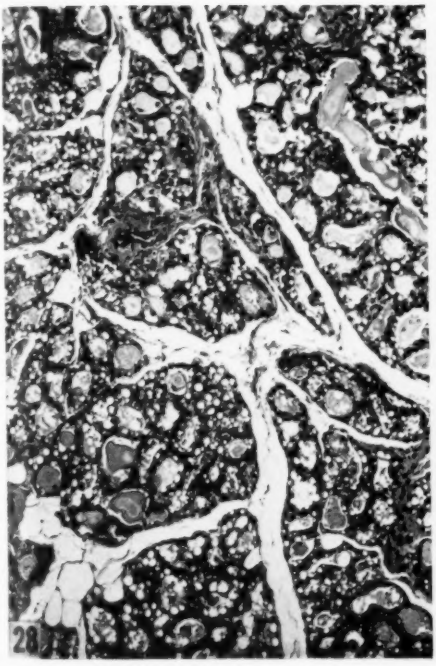
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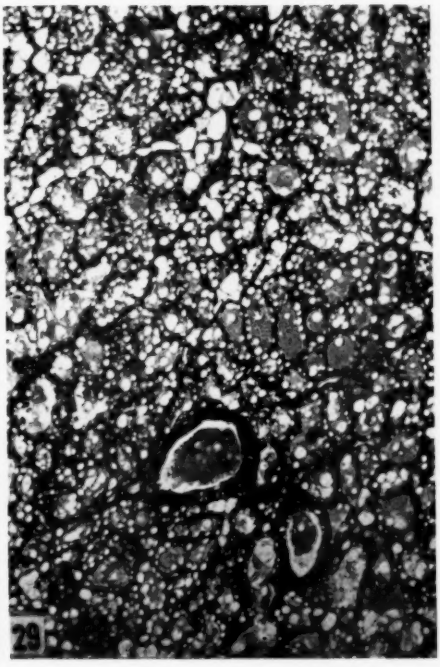
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PLATE 111

FIGURE 30.—Mammary gland of a normal male rat 6 months of age. Hematoxylin and eosin. × 125

FIGURE 31.—Mammary gland of a 6-month-old male rat inoculated 3½ months previously with tumor 494. There is an excessive amount of hyalinized stroma around the acini. Hematoxylin and eosin. × 125

FIGURE 32.—Mammary gland of an 8-month-old male rat inoculated 5 months previously with tumor 494H that had been grown in a female hypophysectomized rat. The ducts and acini are hyperplastic and contain a milklike secretion. Hematoxylin and eosin. × 125

FIGURE 33.—Mammary gland of a 4½-month-old male rat bearing tumor 494NB. The ducts and acini are hyperplastic and contain a milklike secretion. Hematoxylin and eosin. × 125

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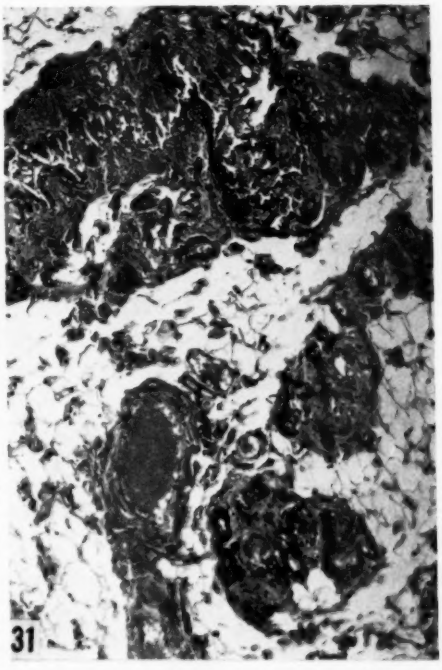
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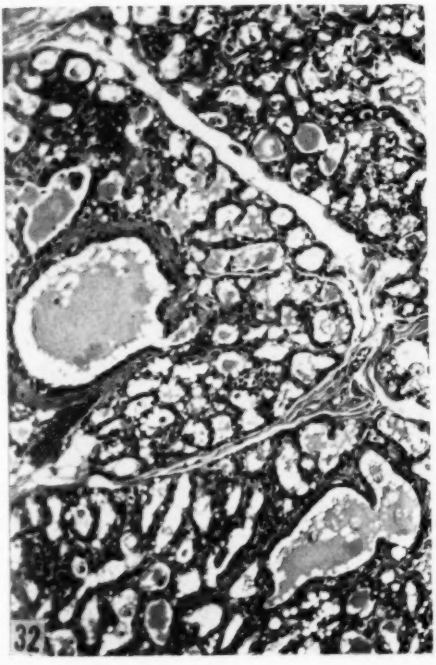
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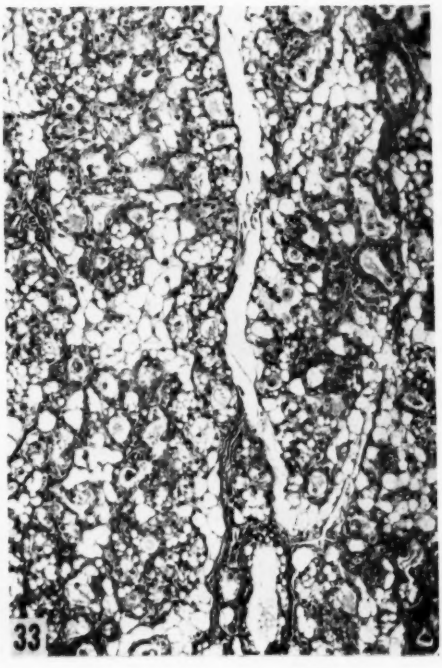
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Uterine-Cancer Morbidity in New York City and Its Relation to the Pattern of Regional Variation Within the United States[1]

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Biometry Branch, National Cancer Institute,[2]
Bethesda, Maryland

Summary

The results of a New York City uterine-cancer morbidity survey are presented. Cervical- and corpus-cancer incidence rates were computed, taking into account age, race, ethnic group, and histologic type. Wide variation in incidence among racial and ethnic groups was noted for cervical cancer but not for corpus cancer. Jewish women had very low cervical-cancer rates. The New York City results were generally consistent with observations reported from other Northern areas of the United States. The collective findings of morbidity surveys and related studies within the United States point to a regional difference in cervical cancer but not in corpus cancer, the cervical rates being higher for white women in the South. Several characteristics known to be associated with cervical cancer—marital status, age at marriage, child-bearing history, socioeconomic status, circumcision of husband, and syphilis—could account for part, but not all, of the regional difference.—**J. Nat. Cancer Inst. 22: 1157–1181, 1959.**

A survey of uterine-cancer morbidity in New York City was undertaken in 1953 and 1954 by the Biometry Branch of the National Cancer Institute at the request of the Laboratory of Pathology of the same Institute. The primary objective was to collect data to assist in interpreting results from the controlled retrospective investigation of this disease that was being conducted in New York City by the latter laboratory. New York has sizable Jewish, Negro, and Puerto Rican populations, and the morbidity survey provided information on the uterine-cancer experience for these groups as well as for the non-Jewish white population. Some of the contrasts of experience among Jewish and non-Jewish white women have been presented and discussed elsewhere (*1*).

The New York City data add to a growing collection of reports on incidence of uterine cancer available from morbidity surveys conducted in 10 metropolitan areas (*2*), Iowa (*3*), Memphis and Shelby County, Tennessee (*4*), and from cancer registers maintained in Connecticut

[1] Received for publication January 14, 1959.
[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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(*5*) and New York State (*6*). This paper presents, systematically, the New York City results, hitherto unpublished, and discusses their relationship to the pattern of geographic variation suggested by a collective review of the other studies.

Materials and Methods

The New York City survey, like that conducted in Memphis, was restricted to one site—the uterus. The procedures essentially followed those employed elsewhere, which are described in reference *2*. Care was taken to distinguish between diagnoses of *in situ* and invasive carcinoma. The hospital records almost invariably specified the location of the lesion within the uterus, with less than 3 percent of the newly diagnosed cases being characterized as uterus and not otherwise specified.

The survey covered New York City residents admitted to a New York City hospital in 1952 and diagnosed for the first time during that year. The hospitals cooperating represented over 97 percent of all hospital beds in the city and included institutions operated by the City Department of Hospitals. Records of private physicians were not surveyed because the prevailing practice in this area is to give radiotherapy for uterine cancer in hospitals and not in offices of radiologists. In the Memphis survey, physicians' records accounted for only 7 percent of the newly diagnosed uterine-cancer cases and it was felt that the proportion would be still smaller in New York City. Cases reported by death certificate only were not included; a preliminary check indicated that the additional number to be found in this manner would be trivial and would increase the total number of diagnosed cases by less than 1 to 2 percent. The expense of a complete review of the death certificates was considered unwarranted.

The populations used for the computation of incidence rates were taken from the 1950 census. Estimates of the Jewish and non-Jewish white populations were derived from a sample of the New York City population studied in 1952 by the Health Insurance Plan of Greater New York, to which we are indebted for unpublished data. With the exception of Puerto Ricans, the use of 1950 population data may be regarded as yielding satisfactory approximations of the incidence rates. Migration of Puerto Ricans into the area was continuing at a rapid rate during the 1950–52 interval and for this reason the incidence rates for Puerto Ricans are undoubtedly overstated. Also, the number of diagnosed cases in the latter group may have been inflated by inclusion of some women diagnosed as having uterine cancer in Puerto Rico before migration to New York City.

Incidence has been defined in the usual manner as the number of new cases diagnosed during the year per 100,000 women. No survey can uncover cases which remain undiagnosed because the patients were never seen by a physician. The age-adjusted rates in tables 3, 5, and 6 are based on the direct method of weighting age-specific rates, with the total 1950 United States Census population as the standard. The

standardized ratios presented in table 4 were computed by the indirect method, with the age-specific rates for the entire city used to compute "expected" numbers for each subgroup.

The New York City rates, while underestimates for reasons set forth, still remain useful for comparisons of experience within the city and with survey and register materials for other areas. In an attempt to improve comparability with results for the 10 metropolitan areas, the rates for the latter have been recomputed, excluding cases reported by death certificate only. This adjustment probably compensates also for the small proportion of cases missed in New York City by noncoverage of private physicians.

Results

The 1,534 cases newly diagnosed in 1952 are distributed by detailed site and diagnostic criteria in table 1. More than 94 percent of the diagnoses were based on microscopic confirmation. Less than 3 percent of the reports failed to specify the cervix or the corpus as the site of the primary lesion, a figure much lower than that usually reported. The crude rates per 100,000 females over 15 years of age are given in the bottom half of table 1. The cervical-cancer rate based on all diagnoses, 26.4, was two fifths greater than the corresponding rate for corpus cancer, 19.3.

Of the 802 microscopically confirmed cases of cervical cancer, 95, or 12 percent, were classified in the records as *in situ*. The number and proportion of diagnoses *in situ* in any survey will, of course, be influenced by the uterine-cancer case-finding procedures used in the area surveyed. In Memphis, where an extensive cytology screening program was under way at the time of the survey, the higher proportion of carcinoma *in situ* of the cervix, 19 percent, reflected in part the effect of the screening pro-

TABLE 1.—*Number of newly diagnosed cases of cancer of the uterus, with rates per 100,000 females 15 years and over, by detailed site and diagnostic criteria, New York City, 1952*

Diagnostic criteria	Total uterus	Cervix	Corpus	Uterus*
	Number			
All diagnosed cases	1,534	862	629	43
Microscopically confirmed	1,444	802	611	31
Invasive	1,337	707	599	31
In situ	107	95	12	0
Not microscopically confirmed	90	60	18	12
	Rate			
All diagnosed cases	47.0	26.4	19.3	1.3
Microscopically confirmed	44.2	24.6	18.7	0.9
Invasive	40.9	21.6	18.3	0.9
In situ	3.3	2.9	0.4	0.0

*Not otherwise specified.

gram in uncovering previously undiagnosed cases *in situ*. Only 12 lesions *in situ* of the corpus were found—2 percent of the total number of corpus-cancer cases in New York City, which is in accord with other impressions of its relatively infrequent diagnosis.

Information on histologic type is contained in table 2. The close association between histologic type and detailed site found in other studies, which provides the basis for the use of histologic type to allocate cases of uterine cancer, unspecified, to the cervix or the corpus, appeared in the New York City data. While reported adenocarcinoma of the cervix is not rare, one may speculate as to whether this group contains some cases where the true primary lesion may be in the corpus rather than in the cervix.

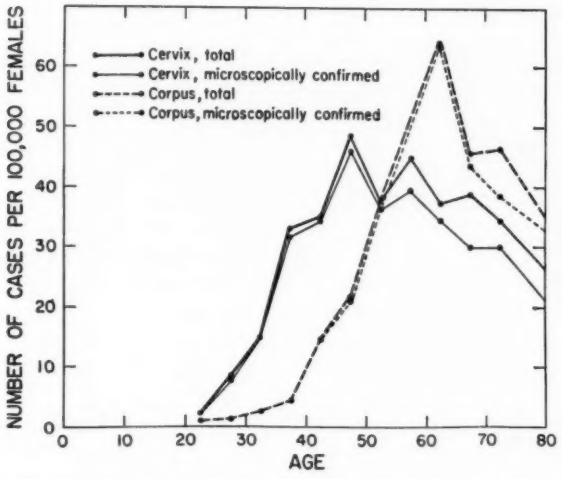
TABLE 2.—*Number of newly diagnosed cases of cancer of the uterus, with microscopic confirmation, by detailed site and histologic type, New York City, 1952*

Histologic type	Total	Cervix	Corpus	Uterus*
Total	1,444	802	611	31
Invasive	1,337	707	599	31
In situ	107	95	12	0
Epidermoid	709	702	3	4
Invasive	614	608	2	4
In situ	95	94	1	0
Adenocarcinoma	596	61	526	9
Invasive	588	60	519	9
In situ	8	1	7	0
Other histologic types†	139	39	82	18
Invasive	135	39	78	18
In situ	4	0	4	0

*Not otherwise specified.

†Includes sarcoma, adenoacanthoma, chorioepithelioma, and carcinoma, undifferentiated and not otherwise specified.

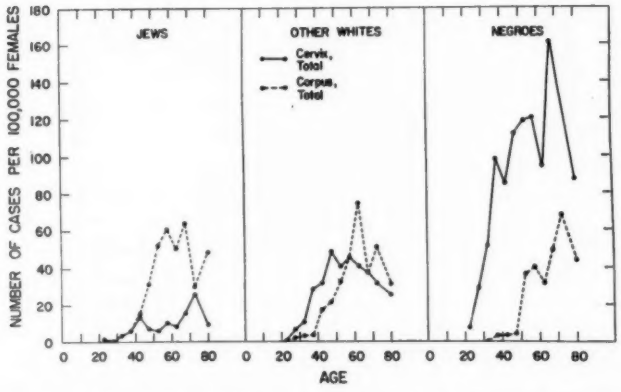
The age-specific incidence rates for cervical and corpus cancer in New York City are shown in text-figure 1. The rates, based on all diagnoses and on microscopically confirmed cases only, yielded essentially the same contrasts between cervix and corpus. Reliance on microscopically confirmed cases alone would accentuate the apparent drop in incidence, at older ages, for both sites, since the proportion of microscopically confirmed cases becomes smaller with advancing age. The New York City results, in which the corpus-cancer rates exceeded those for the cervix after the age of 55 years, were unusual and departed from other experience recorded in the United States. In Memphis, among whites and nonwhites, the cervix rates were higher throughout the entire lifespan. This observation held also for the 4 Southern cities in the 10-city surveys. In the 4 Northern cities and in Connecticut and New York State, exclusive of New York City, the corpus rates more closely approached those for cervix, at older ages, but, in general, did not exceed them. The New York City result cannot be attributed to the presence of a large Jewish popula-



TEXT-FIGURE 1.—Age-specific incidence of cancer of the cervix and corpus, New York City, 1952.

tion as can be seen from text-figure 2, which shows the incidence for Jewish, other white, and Negro women separately. The excess corpus rate after 55 years of age appeared among other white women as well.

The relationship of cervical to corpus cancer peculiar to New York City can be attributed to the low rates there for cervical cancer after 50 years of age. Comparison of New York City (white, non-Jewish women) with the 4 Northern cities (*see* text-fig. 4) showed a not too dissimilar schedule of cervical-cancer rates to 35 years of age, the great divergence occurring after 50 years. Since the New York City corpus-cancer rates, at older ages, resembled those for the Northern cities, the lower cervical incidence



TEXT-FIGURE 2.—Age-specific incidence of cancer of the cervix and corpus among Jewish women, other white women, and Negro women, New York City, 1952.

cannot be ascribed to differences in survey coverage and diagnostic practices.

The age detail in text-figure 2 is summarized in the form of age-adjusted rates in table 3, which includes results for Puerto Rican women as well. To facilitate comparisons with other areas, the United States Census population of 1950 was used as the standard for age-adjustment. The over-all New York City experience concealed wide variation in cervical-cancer incidence among racial and ethnic groups, which was not evident for corpus cancer. If uterine-cancer cases, not otherwise specified, are combined with corpus-cancer cases, on the likely assumption that most of the latter belong in that category, the intergroup variability for corpus cancer is reduced to even smaller dimensions.

The low cervical-cancer rate observed for Jewish women persisted throughout their lifespan, which is consistent with numerous other observations (*7–11*). It is worth re-emphasizing that the cervical-cancer rate for other white women in New York City was generally lower than that reported for whites elsewhere. The unusually low incidence among other white women after the age of 50 years raises the possibility of a cohort effect: that the older women in New York City, the majority of whom are foreign-born or of foreign parentage, were not exposed to some factor now affecting younger women in this city.

The age-specific cervical-cancer incidence started to increase at an earlier age among Negroes than among whites. The New York City-Negro incidence, however, was somewhat lower than that reported from Memphis and from the 4 Southern and 4 Northern cities included in the 10-city surveys. While the Puerto Rican incidence rates may have been inflated by underestimating the population base, there can be little doubt that their rate was at least as high as that for Negroes. The vital statistics data for Puerto Rico (*12*) indicate a high uterine-cancer mortality among the island women and it must be accepted that cervical cancer occurs frequently among them.

The difference in corpus-cancer incidence between Jewish and other white women was small, although the rate for Jewish women was slightly higher. In general, the corpus exhibited a more stable pattern of age-specific incidence by racial and ethnic group. This comment on the relative stability of corpus-cancer incidence applies to comparisons between New York City and other localities as well. The latter observation weighs against attributing the geographic variation in cervical cancer to artifacts arising from survey, diagnostic, and classification procedures.

Table 3 gives 2 ratios of cervix to corpus cancer computed from the age-adjusted rates, one based on cases of specified site and the other incorporating adjustment for cases of unspecified site, following the presentation of Dorn and Cutler (*2*). Since the proportion of unspecified site was small for New York City, the 2 indexes yielded substantially the same inferences. The Jewish ratio, 0.3, was far lower than in other groups, all of which had ratios greater than unity. Other white women had a ratio slightly in excess of 1; for Negroes and Puerto Ricans the

TABLE 3.—*Age-adjusted* incidence rates of cancer of the uterus per 100,000 females by detailed site and diagnostic criteria for 4 racial and ethnic groups, New York City, 1952*

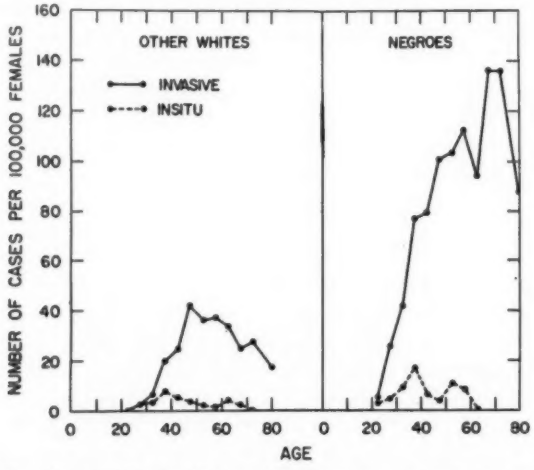
Racial and ethnic groups	All newly diagnosed cases					Microscopically confirmed				Ratio: cervix to corpus	
	Uterus, total		Cervix	Corpus	Uterus†	Cervix		Corpus		Cases with specified site	Adjusted for unspecified site
	Number	Rate				Invasive	*In situ*	Invasive	*In situ*		
Total‡	*1,526*	32.8	18.3	13.6	1.0	14.9	2.0	12.9	0.3	1.35	1.30
Jewish	*277*	20.6	4.7	15.6	0.3	3.6	0.6	15.2	0.2	0.30	0.30
Other white	*901*	31.3	17.1	13.3	0.9	13.5	2.1	12.6	0.3	1.29	1.24
Puerto Rican	*99*	121.1	109.8	7.8	3.6	97.6	5.7	7.8	0.0	14.1	9.7
Negro‡	*249*	67.1	53.6	10.4	3.1	47.8	4.1	9.2	0.4	5.2	4.3

*Rates adjusted to the age distribution of the United States 1950 census population.
†Not otherwise specified.
‡Excludes other nonwhites.

ratios (adjusted for unspecified site) were 4.3 and 9.7, respectively. The cervix-corpus ratio for other white women in New York City equaled the lowest in the series summarized by Dorn and Cutler (Saskatchewan, 1.3). The other most nearly comparable cervix-corpus ratios for whites are found in Upstate New York, 1.6; Connecticut, 1.7; and Iowa, 1.8.

Although invasive cervical carcinoma contributed most of the variation noted for cervical cancer among ethnic groups in New York City, the identical gradient appeared for carcinomas *in situ* as well. The race differential for carcinoma of the cervix *in situ* was also found in Memphis. It is worth re-emphasizing that only a fraction of the lesions *in situ* present in the population come to diagnosis in that stage, so that no significance should be attached to the absolute values of the rates *in situ* presented in this report; the rates can be used for comparative purposes only. Also, the diagnoses *in situ* in New York City reflected the opinions of individual hospital pathologists and were not reviewed by another pathologist using uniform diagnostic criteria. While individual readings might be changed on review, it seems unlikely that the *comparative* findings on an ethnic gradient for lesions *in situ* would be substantially altered by such a review.

Text-figure 3 gives the age-specific incidence rates for carcinoma *in situ* and invasive carcinoma of the cervix for other white and Negro women; the data on carcinoma *in situ* for Jewish women were too scanty for presentation. The results suggested a peak incidence *in situ* at 35 to 39 years among both groups and among other whites, a peak for invasive carcinomas 10 years later, at 45 to 49 years. Among Negroes the incidence of invasive cervical cancer appeared to level off after the age of 55 years. Both the age-relationships and the pattern of variation by ethnic group are consistent with the hypothesis that carcinoma



TEXT-FIGURE 3.—Age-specific incidence of invasive cancer and cancer *in situ* of the cervix among other whites and Negroes, New York City, 1952.

in situ of the cervix may be a precursor stage of invasive cervical cancer, but they do not constitute proof nor can they cast light on the nature of the relationship or on the duration of presymptomatic invasive and *in situ* phases. Further work with repeated cytology screening of large populations to extend the results reported by Dunn (*13*) would be required to establish these points.

Table 4, which is restricted to microscopically confirmed invasive cases, contrasts the observed and the expected numbers of specific histologic types among the 4 ethnic and racial groups. The expected numbers were obtained from the over-all New York City experience and the results are expressed as a standardized ratio of observed to expected numbers of cases. For the total uterus the ethnic-group variation was less pronounced for adenocarcinomas than for epidermoid carcinomas. The Jewish cervical-cancer deficit was concentrated in the epidermoid type. On the other hand, the Negro and Puerto Rican excess of cervical cancer appeared in both adenocarcinoma and epidermoid types. Virtually all the corpus lesions were adenocarcinomas, and consideration of histologic type is not required for them.

While the observations on sarcomas were not extensive, they are in accord with clinical impressions that Jewish women do not experience a lower risk for that histologic type. More data would be required to determine whether the 4 ethnic and racial groups differ in liability to sarcoma. The hint of a lower adenoacanthoma risk for Jewish women, resembling their experience for epidermoid carcinoma, should be interpreted cautiously. No adenoacanthoma diagnoses were reported from the major hospitals operated under Jewish auspices, and this particular deficit may merely reflect a preference for other classifications used by pathologists in these hospitals. While the number of chorioepitheliomas among Puerto Ricans was small, the excess observed seems consistent with what is known about its frequency among persons who live in some tropical areas (*14–16*).

The age-adjusted rates by borough for all white women are shown in table 5. The absence of population data prevented separate examination of interborough variation for Jewish and other white women. The Puerto Rican and Negro populations were concentrated in 1 borough and could not be studied to advantage from this view. The highest rates for cervical and corpus cancer were noted in Manhattan, and differences among the other 4 boroughs were not striking. The cervical-cancer incidence in Manhattan probably reflected the influence of socioeconomic class, since it is well established that the lower socioeconomic classes exhibit substantially higher rates for cervical (*17–19*) but not for corpus cancer. There can be little doubt that if the New York City data had been examined for socioeconomic gradients, results similar to those noted in the 10-city surveys (*17*) would have been found.

The lack of information on marital status, by age, forced consolidation of the rates in table 6 into 2 categories: whites (including Puerto Ricans and nonwhites. The New York City results were consistent with

TABLE 4.—*Standardized ratios * of observed to expected numbers of microscopically confirmed, invasive cases of cancer of the uterus, by histologic type and detailed site for 4 racial and ethnic groups, New York City, 1952*

Histologic type—racial and ethnic group	Total uterus †		Corpus		Cervix	
	Observed	Standardized ratio	Observed	Standardized ratio	Observed	Standardized ratio
All types						
Jewish	257	0. 67	206	1. 17	47	0. 24
Other white	769	0. 93	362	0. 97	392	0. 91
Puerto Rican	88	3. 64	5	0. 54	81	5. 7
Negro	217	2. 2	25	0. 63	182	3. 11
Adenocarcinoma ‡						
Jewish	190	1. 09	185	1. 17	11	0. 64
Other white	362	0. 99	326	0. 98	38	1. 02
Puerto Rican	6	0. 68	3	0. 38	3	2. 71
Negro	29	0. 75	20	0. 58	8	1. 82
Epidermoid §						
Jewish	33	0. 19	0	.....	35	0. 20
Other white	338	0. 90	1	.....	350	0. 89
Puerto Rican	74	5. 8	0	‖	78	6. 0
Negro	165	3. 2	1	.....	174	3. 3
Sarcoma						
Jewish	20	1. 31	19	1. 53	1	.....
Other white	26	0. 77	21	0. 77	3	.....
Puerto Rican	1	0. 91	1	1. 11	0	‖
Negro	6	1. 43	3	0. 88	0	.....
Adenoacanthoma						
Jewish	1	0. 22	1	.....	0	.....
Other white	15	1. 53	14	.....	1	.....
Puerto Rican	0	.....	0	‖	0	‖
Negro	0	‖	0	.....	0	.....

Chorioepithelioma						
Jewish	3	1. 03	1	.....	0	.....
Other white	4	0. 55	0	.....	0	.....
Puerto Rican	3	6. 0	1	‖	0	‖
Negro	2	1. 54	1	.....	0	.....

* The standardized ratios for social and ethnic groups are expressed in terms of the corresponding New York City rates after adjustment for age by the indirect method.

† Includes uterus, not otherwise specified.

‡ Carcinoma, undifferentiated and not otherwise specified, of the corpus, included with adenocarcinoma of the corpus.

§ Carcinoma, undifferentiated and not otherwise specified, of the cervix, included with epidermoid carcinoma of the cervix.

‖ Not computed.

TABLE 5.—*Age-adjusted* incidence rates of cancer of the uterus per 100,000 females, by detailed site, and borough of residence, New York City, whites†, 1952*

Borough	Total uterus	Cervix		Corpus total
		Total	*In situ*	
Total	28.4	13.4	1.6	14.3
Manhattan	35.6	17.6	2.7	17.3
Bronx	24.4	10.6	1.2	12.8
Brooklyn	28.3	12.6	1.4	15.1
Queens	24.2	12.6	1.5	11.1
Richmond	26.7	13.2	0.0	12.5

*Rates adjusted to age distribution of the United States 1950 census population.
†Excludes Puerto Ricans.

numerous observations indicating that the incidence of cervical cancer is higher among married women (*2*). The rate for never-married whites, 8.1, was 49 percent lower than that for the ever-married, and their relative position coincided with that reported in the 10-city survey. The New York City data also revealed a lower rate among never-married nonwhites, contrary to the experience of the 10-city survey. Lesions *in situ* showed the same gradient by marital status as invasive cervical carcinomas. The differences for corpus cancer were smaller than for cervical. The New York City and the 10-city data agreed in suggesting a somewhat higher corpus rate for single white women.

TABLE 6.—*Age-adjusted* incidence rates of cancer of the uterus per 100,000 females, by detailed site, marital status, and race, New York City, 1952*

Marital status	Cervix			Corpus	
	Total	Epidermoid		Total	Adeno-carcinoma
		Invasive	*In situ*		
White†					
Never-married	8.1	5.1	0.5	17.0	14.6
Ever-married	15.8	10.7	1.9	12.8	10.7
Nonwhite					
Never-married	30.2	27.7	1.1	11.2	5.6
Ever-married	57.5	45.5	4.6	10.8	6.5

*Rates adjusted to the age distribution of the United States 1950 census population.
†Includes Puerto Ricans.

Discussion

The results of the morbidity surveys in New York City and Memphis conform to the pattern of regional variation for uterine cancer established by surveys in 10 metropolitan areas and by registration of cancer cases in Upstate New York and Connecticut. The salient features summarized in table 7 are: (*a*) the excess cervical-cancer rates among whites in Southern cities, which run 50 to 60 percent higher than in the North; (*b*) the much narrower North-South differential in cervical cancer

among nonwhites; (*c*) the relatively stable rates for corpus cancer among both whites and nonwhites in all areas. The only finding which might be construed as a deviation from the pattern is the high cervical-cancer rate among residents of urban Iowa. The minor variations in type of coverage and methods for allocating cases of unspecified uterine cancer to cervical or corpus cancer do not affect the general character of the results. It may be noted that distribution of unspecified uterine cancer to cervical or corpus cancer on the basis of histologic-type information yields answers not greatly different from those obtained by combining unspecified uterine cancer with corpus cancer.

TABLE 7.—*Comparisons of age-adjusted* incidence rates of cancer of the uterus observed in several localities*

Localities	Whites		Nonwhites	
	Cervix	Corpus	Cervix	Corpus
	Rates per 100,000 females			
New York City,† 1952	17. 1‡	14. 2‡, §	53. 6	13. 5§
4 Northern cities,† 10-city surveys, 1947 (*20*)	31. 5‖	14. 7‖	72. 4‖	13. 1‖
New York State, exclusive of New York City, urban areas, 1949–51 (*6*)	26. 5¶	17. 7§, ¶	—	—
Connecticut, 1947–51 (*5*)	26. 1‖, ¶	15. 5‖, ¶	—	—
Iowa, urban areas, 1950 (*3*)	46. 8‖, ¶	21. 2‖, ¶	—	—
Memphis, 1950–51 (*4*)	37. 6	14. 6§	64. 1	15. 6§
4 Southern cities,† 10-city surveys, 1947 (*20*)	49. 5‖	16. 1‖	76. 0‖	17. 0‖

*Rate adjusted to age distribution of the United States 1950 census population.
†Excludes cases reported by death certificate only.
‡Excludes Jewish women.
§ Includes other and unspecified uterus.
‖ Uterus, not otherwise specified, distributed to cervix or corpus on the basis of histologic-type information.
¶ Includes a small proportion of nonwhites.

The smaller North-South gradient in cervical cancer among nonwhites, which might have been anticipated on the grounds that many Northern Negroes have migrated from the South, reinforces the significance to be attached to the finding for whites. This feature, coupled with the stable corpus-cancer rates, would appear to rule out an interpretation which ascribes the differences observed to diagnostic factors alone. The available vital statistics data, even though inferior to other materials with respect to quality of diagnostic information and site detail, also point in the same direction. The age-adjusted cervical-cancer mortality (1949–51) per 100,000 white females in the 3 Southern geographic divisions (South Atlantic, East South Central, and West South Central) was 33.4 percent, 19 percent higher than the rate for 3 Northern divisions (New England,

Middle Atlantic, and East North Central), which was 28.0 percent, while the respective rates for corpus and unspecified uterine cancer were virtually identical, 28.2 and 28.6 percent (*21*).

We turn now to the question whether the regional configuration for whites is consistent with other epidemiological characteristics of cervical cancer, a subject recently reviewed by Kaiser and Gilliam (*22*). Table 8, which recapitulates some of the published findings, emphasizes the quantitative aspects of differences in relative risk associated with marital status, number of pregnancies, age at first marriage, socioeconomic status, circumcision of husband, and syphilis.

Survey, register and vital-statistics data point consistently to a slightly greater than twofold risk among married as opposed to single women. The retrospective studies suggest a somewhat greater risk. The relative risks based on marital status may be accepted as a lower limit of the magnitude of the risks to be obtained from contrasts of virgins and nonvirgins, which is supported by the extreme examples of Gagnon's observation of the virtual absence of cervical cancer among nuns (*30*) and Rojel's note of its frequent occurrence among prostitutes (*31*). Despite the magnitude of the effects linked with marital status and sexual intercourse, this factor alone could not be important in reconciling the North-South differences in cervical cancer. The proportion of married women between 35 and 60 years (and presumably of nonvirgins as well) was only slightly higher in urban areas of the South in 1950, 91.8 percent of white females as against 89.4 percent in the North (*32*) and threefold or fourfold or even larger risks for married women could produce only trivial regional differentials in cervical-cancer incidence.

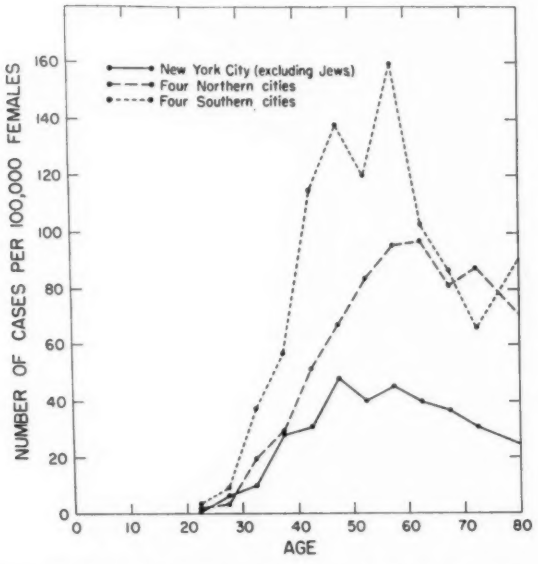
Although birth rates are higher in the South, the regional differences in fertility cannot be attributed to the greater number of children born there. The proportion of childless married women between the ages of 35 and 60 years is almost the same in the urban areas of the North and South, 19.1 and 20.5 percent, respectively. The English vital statistics and most of the retrospective studies suggest that the important distinction for cervical-cancer risk is between fertile and childless women and that an increasing number of pregnancies contributes little or no additional risk. Maliphant is almost alone in reporting greater risks for women bearing several children. It seems evident that the factors of fertility and proportion of childless women could not account for the regional differences in cervical cancer.

Age at first marriage (and presumably age at first intercourse) discriminates between diagnosed cases and controls much more strongly than parity. The three studies listed indicate that the relative risk for women marrying before the age of 20 years is at least twice that for women marrying later. This is an item for which a substantial North-South difference exists. The United States marriage data for 1940 (*33*), the earliest reporting age of bride and that would be descriptive of women now at a risk to cervical cancer, showed that 32.5 percent of the white Southern brides married before the age of 20 years, while the corresponding

figure for Northern brides was 18.7 percent. The North-South difference in early marriage was not so pronounced among Negroes, the respective percentages being 25.5 and 34.9. The risk associated with early marriages could account for a 10 percent higher cervical-cancer incidence among Southern white women. An even larger differential might be predicted if age at first intercourse could be considered.

The association of cervical cancer with age at first marriage is supported by the age pattern of the incidence rates. If the association reflects the operation of some etiologic factor, it would be reasonable to expect groups exposed at an earlier age to evidence responses at an earlier age. Text-figure 4 shows cervical-cancer incidence in 4 Northern cities to lag 6 to 10 years behind that in 4 Southern cities up to 50 years of age. It has already been observed that the New York City incidence at younger ages resembles that prevailing in the other Northern cities, the major divergence appearing after the age of 50 years. The New York City experience on early marriage is similar to that for other Northern cities, so these results are consistent with the relationship just noted. Some other explanation, of course, is still required for the low New York City cervical-cancer incidence at older ages.

The experience for nonwhites conforms to the pattern of early high cervical-cancer incidence among groups in which early marriage is common. The relationship has been found in other cultures as well. Rewell (*34*) has reported, after allowing for age composition of the population, that cervical cancer occurs on the average 10 years earlier in the State of



TEXT-FIGURE 4.—Age-specific incidence of cancer of the cervix among whites, New York City (1952), 4 Northern cities (1947), and 4 Southern cities (1947).

TABLE 8.—*Comparison of reported findings on relative risk of cervical cancer associated with marital status, number of pregnancies, age at first marriage, social class, circumcision of husband, and syphilis*

Reference	Nature of material	Findings
	Marital status	
New York City survey	Survey of diagnosed cases, 1952	Ratio of age-adjusted incidence rates: ever-married to single women, Whites 2.0:1 Nonwhites 1.9:1
Dorn and Cutler (*17*)	Survey of diagnosed cases in 10 metropolitan areas, 1947	Ratio of age-adjusted incidence rates: ever-married to single women, Whites 2.2:1 Nonwhites 1.04:1
Clemmesen (*18*)	Cases reported to Danish Cancer Registry, 1943–47	Graph shows age-specific incidence rates for married women to be double those for single women
Stocks (*19*)	Deaths in England and Wales, 1950–51	Ratio of age-adjusted mortality rates: married to single women, 2.3:1
Logan (*23*)	Deaths in England and Wales, 1948–49	Ratio of age-adjusted mortality rates: married to single women, 2.2:1
Lombard and Potter (*24*)	Retrospective study of cases reported to Massachusetts cancer register, 1946–47	Data suggest a relative risk for married *vs.* single women on order of 4:1
Maliphant (*25*)	Retrospective study in Cardiff, Wales, 1922–46	Data suggest a relative risk for married *vs.* single women on order of 9:1

Number of pregnancies		
Stocks (*19*)	Deaths in England and Wales, 1950–51	Age-adjusted mortality rate higher among married, fertile women. Ratios: married, fertile and married, infertile to single women, 2.4:1 and 2.0:1, respectively
Logan (*23*)	Deaths in England and Wales, 1948–49	Age-adjusted mortality rates higher among married, fertile women. Ratios: married, fertile and married, infertile to single women, 2.3:1 and 1.8:1, respectively
Stocks (*19, 26*)	Retrospective studies in the Liverpool and North Wales area	Number of confinements not found to be associated with cervical-cancer risk for women married under 25 years of age; an association may be present for women married after the age of 25 years
Maliphant (*25*)	Retrospective study in Cardiff, Wales, 1922–46	Data suggest a relative risk for multiparous *vs.* nulliparous women on order of 4:1. Relative risk rises with number of pregnancies
Wynder *et al.* (*27*)	Retrospective study in gynecologic services of several U.S. hospitals	No association between number of pregnancies and cervical cancer
Age at first marriage		
Stocks (*19, 26*)	Retrospective study of cases and deaths in the Liverpool and North Wales area	Data suggest a relative risk for women married before the age of 20 years *vs.* women married after the age of 20 years on order of 2:1
Wynder *et al.* (*27*)	Retrospective study in gynecologic services of several U.S. hospitals	Data suggest a relative risk for women married before the age of 20 years *vs.* women married after the age of 20 years on order of 2:1
Lombard and Potter (*24*)	Retrospective study of cases reported to Massachusetts cancer register, 1946–47	Data suggest a relative risk for women married before the age of 20 years *vs.* women married after the age of 20 years on order of 3:1
Jones *et al.* (*28*)	Retrospective study of cases interviewed in Los Angeles hospitals	Data suggest a relative risk for women married before the age of 20 years *vs.* women married after the age of 20 years on order of 1.5:1

TABLE 8.—*Comparison of reported findings on relative risk of cervical cancer associated with marital status, number of pregnancies, age at first marriage, social class, circumcision of husband, and syphilis*—Continued

Reference	Nature of material	Findings
	Socioeconomic class	
Dorn and Cutler (*17*)	Survey of diagnosed cases in 10 metropolitan areas, 1947	Inverse relationship with socioeconomic class. Ratio: lowest to highest socioeconomic class, 2.1:1
Clemmesen (*18*)	Cases reported to Danish Cancer Registry, 1943–47	Inverse relationship with socioeconomic class. Ratio: lowest to highest socioeconomic class, 2.6:1
Stocks (*19*)	Deaths in England and Wales, 1950–51	Inverse relationship with socioeconomic class. Ratio: lowest to highest socioeconomic class, 1.7:1
	Circumcision of husband	
Wynder *et al.* (*27*)	Retrospective study in gynecologic services of several U.S. hospitals	Data suggest a relative risk for women with circumcised husband *vs.* women with uncircumcised husband on order of 0.4:1
Dunham (*1*)	Retrospective study of cases interviewed in New York City hospitals	No association with circumcision status of spouse found
Jones *et al.* (*28*)	Retrospective study of cases interviewed in Los Angeles hospitals	No association with circumcision status of spouse found
	Syphilis	
Levin *et al.* (*29*)	Cases reported to New York State cancer register matched against register of reported syphilis cases	Data suggest a relative risk for women with a history of syphilis *vs.* no history of syphilis on order of 3.5:1
Wynder *et al.* (*27*)	Retrospective study in gynecologic services of several U.S. hospitals	Data suggest a relative risk for women with a history of syphilis *vs.* no history of syphilis on order of 2:1
Lombard and Potter (*24*)	Retrospective study of cases reported to Massachusetts cancer register, 1946–47	Data suggest a relative risk for women with a history of syphilis *vs.* no history of syphilis on order of 4:1

Madras (Southern India) than in England and that age at marriage in Madras occurs about 9 years earlier.

On the other hand, late marriage cannot account for the low incidence among Jewish women. While Wynder's report of a smaller proportion of first marriages under 20 years of age for Jewish controls in his study (*27*) is supported by observations of a higher median age at first marriage among Jewish women in Iowa (*35*), the difference between Jewish and other white women in this regard is not large enough to effect a noticeable reconciliation in their cervical-cancer rates.

Cervical cancer is inversely related to socioeconomic status, and the available data agree that the rate for the lowest socioeconomic class is about double that for the highest class. Since age at marriage is associated with socioeconomic class (*36*), these two findings are not independent and it would be difficult to deduce from data at hand how much of the social-class effect would remain after control for age at marriage. Residents of Southern cities have lower incomes and greater representation of occupations lower in the socioeconomic scale (*37*), so that the presence of higher cervical-cancer incidence there is at least compatible with this feature. This connection between geographic variation and social-class differences has been observed in England. The standardized mortality ratio for cervical-cancer mortality in the south of England for 1950–54 was 40 percent under that in the north of England (*38*), where a larger proportion of persons belong to the lower socioeconomic classes.

The Dorn-Cutler material on variation by income class is based on an alignment of census tracts in each city into five groups, each containing approximately the same number of people, with median incomes and rentals as the criteria for classification. Table 9 contrasts the cervical incidence rates, derived from their data, for white females in Northern and Southern cities (*20*). The Southern rates appear to be displaced by about one income class, the rate for the highest class resembling that for the second highest class in Northern cities, etc. Undoubtedly, statistical control of socioeconomic class would diminish the apparent differences in incidence between Northern and Southern cities. It is questionable whether the North-South differences could be accounted for completely in terms of the complex of factors linked with social class, since this would require that persons in a given income class in the South possess a variety

TABLE 9.—*Age-adjusted*,† incidence rates of cancer of the uterine cervix per 100,000 white females by income class—10-city surveys, 1947*

Income class	4 Northern cities	4 Southern cities
5 (high)	19. 9	31. 4
4	27. 7	31. 8
3	26. 2	43. 6
2	33. 3	50. 9
1 (low)	47. 0	76. 3

*Rates adjusted by indirect method to age distribution of the United States 1950 census population.

†Excludes cases reported by death certificate only.

of personal characteristics resembling those for persons in the next lower income class in the North.

An association with circumcision status of the husband cannot be accepted unreservedly. Wynder's finding of a higher risk for women with uncircumcised husbands rests on rather small numbers, while the studies of Dunham and Jones *et al.* have failed to detect a relationship. Inaccuracies in responses to questions on circumcision status may have diluted beyond recognition an association of cervical cancer with this factor. Lilienfeld and Graham (*39*) compared the history and the examination findings for 213 male patients admitted to Roswell Park Memorial Institute and found disagreement between examination and history in 34 percent. Even larger errors of response and classification might be anticipated when the information is reported by the female partner. It remains to be seen whether significant associations can be detected in studies eliciting accurate data on circumcision status. While the probably higher proportion of circumcised males in Northern States would be in a direction consistent with the regional variation, the small proportion of non-Jewish circumcised males in Wynder's control data suggests that this factor could not account, in any event, for an important part of the regional difference in cervical-cancer incidence.

While studies by Levin and others have revealed higher estimated risks for women with a history of syphilis, some reservations have been expressed on their interpretation. Apart from the issue of adequacy and reliability of the history of syphilis, Wynder has pointed out that prevalence of syphilis is associated with early marriage and that, in his data, this association alone could produce an apparent twofold excess risk of cervical cancer among women with syphilis. While the Levin study (in which the association was specific for cervix) would underenumerate syphilis cases in the cervical-cancer and control series, the method would be adequate for comparative purposes, since the cervix cases and other cancer sites used as controls were uniformly checked against the Upstate New York syphilis register. Because of the inverse relationship of cervical cancer with socioeconomic class, more complete syphilis case reporting for persons in lower socioeconomic classes could produce a small apparent association between syphilis and cervical cancer, but not a relative risk as large as 3 to 1.

Since age at first marriage and the other items listed in table 8 are unlikely to reconcile completely the North-South difference in cervical-cancer incidence, the over threefold relative risk associated with syphilis might be entertained as a factor contributing to the regional difference. Serologic tests of Selective Service examinees (*40*) yield 1 index of regional differences in syphilis prevalence among white males; 1.0 percent in Northern States and 2.7 percent in Southern States. Scattered fragmentary data from prenatal serologic tests point to similar regional differences among white females. However, the population affected is again so small that only a fraction of the regional difference in cervical incidence could be accounted for in this fashion. A better reconciliation

might be effected if the association with syphilis were not a direct one, but reflected indirect associations with other conditions affecting the female genital tract. The racial difference in syphilis prevalence is well known and this would fit in with the observation of higher cervical-cancer rates among Negroes.

While the regional variation in cervical-cancer incidence is not inconsistent with other epidemiological characteristics of this disease, it seems unlikely that the regional differences can be accounted for completely in terms of these other known factors. Many of the items which have been shown to be associated with this disease—marital status, age at marriage, socioeconomic class, and prevalence of syphilis—are closely interrelated, do not yield greatly different magnitudes of relative risks, and appear to lead to no clear-cut choice of hypotheses on causation, although they can be regarded as helpful in narrowing the field of search. Studies of regional variation, by providing another dimension for investigation, can be helpful in further testing and sorting out hypotheses. It may prove a useful counterpoise to ethnic-group comparisons. For example, it seems clear that circumsion of the spouse could not account for regional variation within the United States; on the other hand, a higher age at first marriage could not be responsible for the very low incidence among Jewish women.

Appendix A

Population, number of newly diagnosed cases of cancer of the uterus and age-specific rates per 100,000 females, by detailed site and diagnostic criteria for racial and ethnic groups, New York City, 1952

New York City*	All ages†	Under 20 years	20–24	25–29	30–34	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74	75 years and over
Population	4, 078, 525	1, 051, 865	318, 595	350, 775	350, 675	349, 680	333, 635	301, 155	280, 020	225, 585	182, 200	146, 960	93, 130	94, 250
Number of cases‡	1, 534	4	11	34	64	132	171	220	213	224	195	126	76	64
Cervix, total	862	1	7	29	53	115	118	148	106	101	69	58	32	25
micr. conf.§	802	1	7	27	53	111	115	140	102	89	64	45	28	20
invasive	707	1	4	18	41	85	99	129	96	86	58	42	28	20
in situ	95	—	3	9	12	26	16	11	6	3	6	3	—	—
Corpus, total	629	1	3	4	9	15	48	66	106	117	117	67	43	33
micr. conf.	611	1	3	4	9	15	48	64	104	116	116	64	36	31
Rates	37.6	0.4	3.5	9.7	18.3	37.8	51.3	73.1	76.1	99.3	107.0	85.7	81.6	67.9
Cervix, total	21.1	0.1	2.2	8.3	15.1	32.9	35.4	49.2	37.9	44.8	37.9	39.5	34.4	26.5
micr. conf.	19.6	0.1	2.2	7.7	15.1	31.7	34.5	46.5	36.4	39.4	35.1	30.6	30.1	21.2
invasive	17.3	0.1	1.3	5.1	11.7	24.3	29.7	42.8	34.3	38.1	31.8	28.6	30.1	21.2
in situ	2.3	—	0.9	2.6	3.4	7.4	4.8	3.7	2.1	1.3	3.3	2.0	—	—
Corpus, total	15.4	0.1	0.9	1.1	2.6	4.3	14.4	21.9	37.9	51.9	64.2	45.6	46.2	35.0
micr. conf.	15.0	0.1	0.9	1.1	2.6	4.3	14.4	21.3	37.1	51.4	63.7	43.5	38.7	32.9
Jewish population‖	1, 047, 000	233, 300	74, 100	82, 100	88, 400	89, 800	93, 200	81, 800	82, 400	67, 400	61, 400	45, 500	26, 800	20, 800
Number of cases‡	277	—	1	—	6	13	28	32	48	48	37	37	15	12
Cervix, total	61	—	—	—	3	6	13	6	5	7	5	7	7	2
micr. conf.	55	—	—	—	3	5	13	6	5	7	3	6	5	2
Corpus, total	212	—	1	—	3	6	14	26	43	41	31	29	8	10
micr. conf.	208	—	1	—	3	6	14	25	42	41	31	28	7	10
Rates	26.5	—	1.4	—	6.8	14.5	30.0	39.1	58.2	71.2	60.3	81.4	56.1	57.8
Cervix, total	5.8	—	—	—	3.4	6.7	14.0	7.3	6.1	10.4	8.2	15.4	26.2	9.6
micr. conf.	5.3	—	—	—	3.4	5.6	14.0	7.3	6.1	10.4	4.9	13.2	18.7	9.6
Corpus, total	20.3	—	1.4	—	3.4	6.7	15.0	31.8	52.2	60.8	50.5	63.8	29.9	48.1
micr. conf.	19.9	—	1.4	—	3.4	6.7	15.0	30.6	51.0	60.8	50.5	61.6	26.2	48.1
White non-Jewish population¶	2, 489, 595	654, 865	186, 440	205, 506	205, 349	206, 827	198, 528	186, 205	173, 800	142, 686	109, 179	91, 305	60, 950	67, 955
Number of cases‡	901	1	3	18	28	66	95	130	126	139	133	68	51	43
Cervix, total	495	—	1	13	21	59	62	90	70	65	44	34	19	17
micr. conf.	451	—	1	11	21	57	59	85	67	55	42	24	17	12

Cervix, total	495	—	1	13	21	59	62	90	70	65	44	34	19	17
micr. conf.	451	—	1	11	21	57	59	85	67	55	42	24	17	12
invasive	392	—	1	5	14	41	49	78	63	53	37	22	17	12
in situ	59	—	—	6	7	16	10	7	4	2	5	2	—	—
Corpus, total	382	—	1	4	6	7	33	38	56	70	81	34	31	21
micr. conf.	371	—	1	4	6	7	33	37	56	70	80	32	26	19
Rates	36.2	.2	1.6	8.8	13.6	31.9	47.9	69.8	72.5	97.4	121.8	74.4	83.6	63.3
Cervix, total	19.9	—	0.5	6.3	10.2	28.5	31.2	48.3	40.3	45.6	40.3	37.2	31.2	25.0
micr. conf.	18.2	—	0.5	5.3	10.2	27.5	29.7	45.7	38.6	38.6	38.5	26.3	27.9	17.7
invasive	15.8	—	0.5	2.4	6.8	19.8	24.7	41.9	36.3	37.2	33.9	24.1	27.9	17.7
in situ	2.4	—	—	2.9	3.4	7.7	5.0	3.8	2.3	1.4	4.6	2.2	—	—
Corpus, total	15.4	—	0.5	2.0	2.9	3.4	16.6	20.4	32.2	49.1	74.2	37.2	50.8	30.9
micr. conf.	14.9	—	0.5	2.0	2.6	3.4	16.6	19.9	32.2	49.1	73.3	35.0	42.7	28.0
Puerto Rican population**	131, 580	49, 240	17, 080	14, 300	12, 990	10, 724	8, 576	5, 954	4, 146	2, 861	1, 959	1, 970	905	875
Number of cases‡	99	1	4	2	6	11	14	20	8	14	12	3	1	3
Cervix, total	92	—	3	2	6	10	14	20	8	14	10	3	—	2
Corpus, total	5	—	1	—	—	1	—	—	—	—	2	—	1	—
Rates	75.2	2.0	23.4	14.0	46.2	102.6	163.3	335.9	193.0	489.3	612.6	152.3	110.5	342.9
Cervix, total	69.9	—	17.6	14.0	46.2	93.3	163.3	335.9	193.0	489.3	510.5	152.3	—	228.6
Corpus, total	3.8	—	5.9	—	—	9.3	—	—	—	—	102.1	—	110.5	—
Negro population††	401, 840	111, 485	39, 840	47, 674	43, 071	41, 634	32, 871	26, 826	19, 369	12, 483	9, 527	8, 075	4, 415	4, 570
Number of cases‡	249	2	3	14	23	42	33	35	31	22	12	17	9	6
Cervix, total	208	1	3	14	22	40	28	30	23	15	9	13	6	4
micr. conf.	202	1	3	14	22	39	28	28	22	15	9	11	6	4
invasive	182	1	2	12	18	32	26	27	20	14	9	11	6	4
in situ	20	—	1	2	4	7	2	1	2	1	—	—	—	—
Corpus, total	28	1	—	—	—	1	1	1	7	5	3	4	3	2
micr. conf.	26	1	—	—	—	1	1	1	6	5	3	4	2	2
Rates	62.0	1.8	7.5	29.4	53.4	100.9	100.4	130.5	160.1	176.2	126.0	210.5	203.9	131.3
Cervix, total	51.8	0.9	7.5	29.4	51.1	96.1	85.2	111.8	118.8	120.2	94.5	161.0	135.9	87.5
micr. conf.	50.3	0.9	7.5	29.4	51.1	93.7	85.2	104.4	113.6	120.2	94.5	136.2	135.9	87.5
invasive	45.3	0.9	5.0	25.2	41.8	76.9	79.1	100.7	103.3	112.2	94.5	136.2	135.9	87.5
in situ	5.0	—	2.5	4.2	9.3	16.8	6.1	3.7	10.3	8.0	—	—	—	—
Corpus, total	7.0	0.9	—	—	—	2.4	3.0	3.7	36.1	40.1	31.5	49.5	68.0	43.8
micr. conf.	6.5	0.9	—	—	—	2.4	3.0	3.7	31.0	40.1	31.5	49.5	45.3	43.8

*Includes "other nonwhites."

†Rates for all ages not age-adjusted.

‡Includes uterus, not otherwise specified.

§Microscopically confirmed.

‖Estimated from sample of New York City population studied in 1952 by the Health Insurance Plan of Greater New York.

¶Obtained by subtracting estimated Jewish and white Puerto Rican populations from total white population.

**Populations for 5-year age groups estimated from published 10-year age groups by use of graduation formulas.

††Excludes Puerto Ricans.

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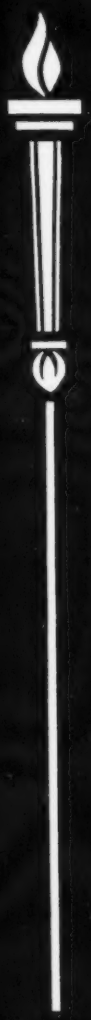

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